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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : GRAPEVINE LEAFROLL VIRUS PROTEINS AND THEIR USES

GRAPEVINE LEAFROLL VIRUS PROTEINS AND THEIR USES

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FIELD OF THE INVENTION

The present invention relates to grapevine leafroll virus proteins, DNA molecules encoding these proteins, and their uses.

BACKGROUND OF THE INVENTION

The world's most widely grown fruit crop, the grape (*Vitis sp.*), is cultivated on all continents except Antarctica. However, major grape production centers are in European countries (including Italy, Spain, and France), which constitute about 70% of the world grape production (Mullins et al., Biology of the Grapevine, Cambridge, U.K.:University Press (1992)). The United States, with 300,000 hectares of grapevines, is the eighth largest grape grower in the world. Although grapes have many uses, a major portion of grape production (~80%) is used for wine production. Unlike cereal crops, most of the world's vineyards are planted with traditional grapevine cultivars, which have been perpetuated for centuries by vegetative propagation. Several important grapevine virus and virus-like diseases, such as grapevine leafroll, corky bark, and Rupestris stem pitting, are transmitted and spread

through the use of infected vegetatively propagated materials. Thus, propagation of certified, virus-free materials is one of the most important disease control measures. Traditional breeding for disease resistance is difficult due to the highly heterozygous nature and outcrossing behavior of grapevines, and due to polygenic patterns of inheritance. Moreover, introduction of a new cultivar may be prohibited by custom or law. Recent biotechnology developments have made possible the introduction of special traits, such as disease resistance, into an established cultivar without altering its horticultural characteristics.

Many plant pathogens, such as fungi, bacteria, phytoplasmas, viruses, and nematodes can infect grapes, and the resultant diseases can cause substantial losses in production (Pearson et al., Compendium of Grape Diseases, American Phytopathological Society Press (1988)). Among these, viral diseases constitute a major hindrance to profitable growing of grapevines. About 34 viruses have been isolated and characterized from grapevines. The major virus diseases are grouped into: (1) the grapevine degeneration caused by the fanleaf nepovirus, other European nepoviruses, and American nepoviruses, (2) the leafroll complex, and (3) the rugose wood complex (Martelli, ed., Graft Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, FAO, UN, Rome, Italy (1993)).

Of the major virus diseases, the grapevine leafroll complex is the most widely distributed throughout the world. According to Goheen (Goheen, "Grape Leafroll," in Frazier et al., eds., Virus Diseases of Small Fruits and Grapevines (A Handbook), University of California, Division of Agricultural Sciences, Berkeley, Calif, USA, pp. 209-212 (1970) ("Goheen (1970)"), grapevine leafroll-like disease was described

as early as the 1850s in German and French literature. However, the virus nature of the disease was first demonstrated by Scheu (Scheu, "Die Rollkrankheit des Rebstockes (Leafroll of grapevine)," D. D. Weinbau 14:222-358 (1935) ("Scheu (1935)")). In 1946, Harmon and Snyder (Harmon et al., "Investigations on the Occurrence, Transmission, Spread and Effect of 'White' Fruit Colour in the Emperor Grape," Proc. Am. Soc. Hort. Sci. 74:190-194 (1946)) determined the virus nature of White Emperor disease in California. It was later proven by Goheen et al. (Goheen et al., "Leafroll (White Emperor Disease) of Grapes in California, Phytopathology, 48:51-54 (1958) ("Goheen (1958)")) that both leafroll and "White Emperor" diseases were the same, and only the name "leafroll" was retained.

Leafroll is a serious virus disease of grapes and occurs wherever grapes are grown. This wide distribution of the disease has come about through the propagation of diseased vines. It affects almost all cultivated and rootstock varieties of *Vitis*. Although the disease is not lethal, it causes yield losses and reduction of sugar content. Scheu estimated in 1936 that 80 per cent of all grapevines planted in Germany were infected (Scheu, Mein Winzerbuch, Berlin:Reichsnahrstand-Verlags (1936)). In many California wine grape vineyards, the incidence of leafroll (based on a survey of field symptoms conducted in 1959) agrees with Scheu's initial observation in German vineyards (Goheen et al., "Studies of Grape Leafroll in California," Amer. J. Enol. Vitic., 10:78-84 (1959)). The current situation on leafroll disease does not seem to be any better (Goheen, "Diseases Caused by Viruses and Viruslike Agents," The American Phytopathological Society, St. Paul, Minnesota:APS Press, 1:47-54 (1988) ("Goheen (1988)"). Goheen also estimated that the disease causes an annual

loss of about 5-20 per cent of the total grape production (Goheen (1970) and Goheen (1988)). The amount of sugar in individual berries of infected vines is only about 1/2 to 2/3 that of berries from noninfected vines (Goheen (1958)).

Symptoms of leafroll disease vary considerably depending upon the cultivar, environment, and time of the year. On red or dark-colored fruit varieties, the typical downward rolling and interveinal reddening of basal, mature leaves is the most prevalent in autumn; but not in spring or early summer. On light-colored fruit varieties however, symptoms are less conspicuous, usually with downward rolling accompanied by interveinal chlorosis. Moreover, many infected rootstock cultivars do not develop symptoms. In these cases, the disease is usually diagnosed with a woody indicator indexing assay using *Vitis vivifera* cv. Carbernet Franc (Goheen (1988)).

Ever since Scheu demonstrated that leafroll was graft transmissible, a virus etiology has been suspected (Scheu (1935)). Several virus particle types have been isolated from leafroll diseased vines. These include potyvirus-like (Tanne et al., "Purification and Characterization of a Virus Associated with the Grapevine Leafroll Disease," Phytopathology, 67:442-447 (1977)), isometric virus-like (Castellano et al.,"Virus-like Particles and Ultrastructural Modifications in the Phloem of Leafroll-affected Grapevines," Vitis, 22:23-39 (1983) ("Castellano (1983)") and Namba et al., "A Small Spherical Virus Associated with the Ajinashika Disease of Koshu Grapevine, Ann. Phytopathol. Soc. Japan, 45:70-73 (1979)), and closterovirus-like (Namba, "Grapevine Leafroll Virus, a Possible Member of Closteroviruses, Ann. Phytopathol. Soc. Japan, 45:497-502 (1979)) particles. In recent years, however, long flexuous closteroviruses ranging from 1,400 to 2,200 nm have been

most consistently associated with leafroll disease (Figure 1) (Castellano (1983), Faoro et al., "Association of a Possible Closterovirus with Grapevine Leafroll in Northern Italy," Riv. Patol. Veg., Ser IV, 17:183-189 (1981), Gugerli et al., "L'enroulement de la vigne: mise en évidence de particules virales et développement d'une méthode immuno-enzymatique pour le diagnostic rapide (Grapevine Leafroll: Presence of Virus Particles and Development of an Immuno-enzyme method for Diagnosis and Detection)," Rev. Suisse Viticult. Arboricult. Hort., 16:299-304 (1984) ("Gugerli (1984)"), Hu et al., "Characterization of Closterovirus-like Particles Associated with Grapevine Leafroll Disease," J. Phytopathol., 128:1-14 (1990) ("Hu (1990)"), Milne et al., "Closterovirus-like Particles of Two Types Associated with Diseased Grapevines," Phytopathol. Z., 110:360-368 (1984), Zee et al., "Cytopathology of Leafroll-diseased Grapevines and the Purification and Serology of Associated Closteroviruslike Particles," Phytopathology, 77:1427-1434 (1987) ("Zee (1987)"), and Zimmermann et al., "Characterization and Serological Detection of Four Closterovirus-like Particles Associated with Leafroll Disease on Grapevine," J. Phytopathol., 130:205-218 (1990) ("Zimmermann (1990)")). These closteroviruses are referred to as grapevine leafroll associated viruses ("GLRaV"). At least six serologically distinct types of GLRaV's (GLRaV-1 to -6) have been detected from leafroll diseased vines (Table 1) (Boscia et al., "Nomenclature of Grapevine Leafroll-associated Putative Closteroviruses, Vitis, 34:171-175 (1995) ("Boscia (1995)") and (Martelli, "Leafroll," pp. 37-44 in Martelli, ed., Graft Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, FAO, Rome Italy, (1993) ("Martelli I")). The first five of these were confirmed in the 10th Meeting of the

International Council for the Study of Virus and Virus Diseases of the Grapevine ("ICVG") (Volos, Greece, 1990).

TABLE 1

Type	Particle length (nm)	Coat protein Mr ($X10^3$)	Reference
GLRaV-1	1,400-2,200	39	Gugerli (1984)
GLRaV-2	1,400-1,800	26	Gugerli (1984) Zimmermann (1990)
GLRaV-3	1,400-2,200	43	Zee (1987)
GLRaV-4	1,400-2,200	36	Hu (1990)
GLRaV-5	1,400-2,200	36	Zimmermann (1990)
GLRaV-6	1,400-2,200	36	Gugerli (1993)

Through the use of monoclonal antibodies, however, the original GLRaV II described in Gugerli (1984) has been shown to be an apparent mixture of at least two components, IIa and IIb (Gugerli et al., "Grapevine Leafroll Associated Virus II Analyzed by Monoclonal Antibodies," 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, Montreux, Switzerland, pp. 23-24 (1993) ("Gugerli (1993)")). Recent investigation with comparative serological assays (Boscia (1995)) demonstrated that the IIb component of cv. Chasselas 8/22 is the same as the GLRaV-2 isolate from France (Zimmermann (1990)) which also include the isolates of grapevine corky bark associated closteroviruses from Italy (GCBaV-BA) (Boscia (1995)) and from the United States (GCBaV-NY) (Namba et al., "Purification and Properties of Closterovirus-like Particles Associated with Grapevine Corky Bark Disease," Phytopathology, 81:964-970 (1991) ("Namba (1991)")). The IIa component of cv. Chasselas 8/22 was given the provisional name of

grapevine leafroll associated virus 6 (GLRaV-6). Furthermore, the antiserum to the CA-5 isolate of GLRaV-2 produced by Boscia et al. (Boscia et al., "Characterization of Grape Leafroll Associated Closterovirus (GLRaV) Serotype II and Comparison with GLRaV Serotype III," Phytopathology, 80:117 (1990)) was shown to contain antibodies to both GLRaV-2 and GLRaV-1, with a prevalence of the latter (Boscia (1995)).

Several shorter closteroviruses (particle length 800 nm long) have also been isolated from grapevines. One of these, called grapevine virus A ("GVA") has also been found associated, though inconsistently, with the leafroll disease (Agran et al., "Occurrence of Grapevine Virus A (GVA) and Other Closteroviruses in Tunisian Grapevines Affected by Leafroll Disease," Vitis, 29:43-48 (1990), Conti, et al., "Closterovirus Associated with Leafroll and Stem Pitting in Grapevine," Phytopathol. Mediterr., 24:110-113 (1985), and Conti et al., "A Closterovirus from a Stem-pitting-diseased Grapevine," Phytopathology, 70:394-399 (1980)). The etiology of GVA is not really known; however, it appears to be more consistently associated with rugose wood *sensu lato* (Rosciglione at al., "Maladies de l'enroulement et du bois strié de la vigne: analyse microscopique et sérologique (Leafroll and Stem Pitting of Grapevine: Microscopical and Serological Analysis)," Rev. Suisse Vitic Arboric. Hortic., 18:207-211 (1986) ("Rosciglione (1986)"), and Zimmermann (1990)). Moreover, another short closterovirus (800 nm long) named grapevine virus B ("GVB") has been isolated and characterized from corky bark-affected vines (Boscia et al., "Properties of a Filamentous Virus Isolated from Grapevines Affected by Corky Bark, Arch. Virol., 130:109-120 (1993) and Namba (1991)).

As suggested by Martelli I, leafroll symptoms may be induced by more than one virus or they may be simply a general plant physiological response to invasion by an array of phloem-inhabiting viruses. Evidence accumulated in the last 15 years strongly favors the idea that grapevine leafroll is induced by one (or a complex) of long closteroviruses (particle length 1,400 to 2,200 nm).

Grapevine leafroll is transmitted primarily by contaminated scions and rootstocks. However, under field conditions, several species of mealybugs have been shown to be the vector of leafroll (Engelbrecht et al., "Transmission of Grapevine Leafroll Disease and Associated Closteroviruses by the Vine Mealybug Planococcus-ficus," Phytophylactica, 22:341-346 (1990), Rosciglione, et al., "Transmission of Grapevine Leafroll Disease and an Associated Closterovirus to Healthy Grapevine by the Mealybug Planococcus ficus," (Abstract), Phytoparasitica, 17:63-63 (1989), and Tanne, "Evidence for the Transmission by Mealybugs to Healthy Grapevines of a Closter-like Particle Associated with Grapevine Leafroll Disease," Phytoparasitica, 16:288 (1988)). Natural spread of leafroll by insect vectors is rapid in various parts of the world. In New Zealand, observations of three vineyards showed that the number of infected vines nearly doubled in a single year (Jordan et al., "Spread of Grapevine Leafroll and its Associated Virus in New Zealand Vineyards," 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, Montreux, Switzerland, pp. 113-114 (1993)). One vineyard became 90% infected 5 years after GLRaV-3 was first observed. Prevalence of leafroll worldwide may increase as chemical control of mealybugs becomes more difficult due to the unavailability of effective insecticides.

In view of the serious risk grapevine leafroll virus poses to vineyards and the absence of an effective treatment of it, the need to prevent this affliction continues to exist. The present invention is directed to overcoming this deficiency in the art.

SUMMARY OF INVENTION

The present invention relates to an isolated protein or polypeptide corresponding to a protein or polypeptide of a grapevine leafroll virus. The encoding RNA and DNA molecules, in either isolated form or incorporated in an expression system, a host cell, or a transgenic *Vitis* or citrus scion or rootstock cultivar, are also disclosed.

Another aspect of the present invention relates to a method of imparting grapevine leafroll virus resistance to *Vitis* scion or rootstock cultivars by transforming them with a DNA molecule encoding the protein or polypeptide corresponding to a protein or polypeptide of a grapevine leafroll virus. These DNA molecules can also be used in transformation of citrus scion or rootstock cultivar to impart tristeza virus resistance to such cultivars.

The present invention also relates to an antibody or binding portion thereof or probe which recognizes the protein or polypeptide.

Grapevine leafroll virus resistant transgenic variants of the current commercial grape cultivars and rootstocks allows for more complete control of the virus while retaining the varietal characteristics of specific cultivars. Furthermore, these variants permit control of GLRaV transmitted either by contaminated scions or rootstocks or by GLRaV-carrying mealy bugs. With respect to the latter mode of transmission, the present invention

circumvents increased restriction of pesticide use which has made chemical control of mealy bug infestations increasingly difficult. In this manner, as well as others, the interests of the environment and the economics of grape cultivation and wine making are all benefited by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is electron micrographs of GLRaV-3 particles of the NY1 isolate after negative staining with 1% uranyl acetate of a purified virus preparation (magnification 80,000X).

Figure 2 shows the nucleotide and amino acid sequences of a PCR amplified fragment of the GLRaV-3 genome. The external and internal primers used for PCR are underlined and their orientations are indicated by arrows.

Figure 3 compares the alignment of the amino acid sequence deduced from the PCR fragment of GLRaV-3 with respective regions of HSP90 homologues of beet yellow virus ("BYV") (p64), citrus tristeza virus ("CTV") (p61), and lettuce infectious yellow virus ("LIYV") (p59). Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids, lowercase letters indicate at least three identical or functionally similar amino acids.

Figure 4, panel B, is a Northern blot hybridization. Probe made from a clone insert gave positive reaction to itself (lane 3) as well as dsRNA from leafroll infected tissues (lane 1), but not to nucleic acids extracted from healthy grapevines (lane 2). Lane M contains a molecular weight marker (the Hind III digested fragments of lambda DNA). Panel A of Figure 4

depicts an ethidium bromide stained agarose gel before transfer to a membrane.

Figure 5 presents an analysis of GLRaV-3 dsRNA by electrophoresis on an ethidium bromide stained agarose gel. A dsRNA of ca. 16 kb was readily isolated from diseased grapevine (lane 6), but not from the healthy control (lane 5). Other samples that were used for control were tobacco mosaic virus dsRNA (lane 1); cucumber mosaic virus dsRNA (lane 2); pBluescript vector (lane 3) and an insert of clone pC4. λ *Hind* III digested fragment of lambda DNA was used as the molecular weight marker (lane M).

Figure 6 is a secondary immunoscreening of plaques derived from three mother plaques that reacted to GLRaV-3 specific polyclonal antibody. Two filters each represent plaques from clones pCP5 (left), pCP8-4 (middle), and pCP10-1 (right).

Figure 7 is a PCR analysis of immuno-positive clones with flanking vector primer (KS and SK). A similar size (1.0-1.1 kb) PCR product was produced in all three mother clones.

Figure 8 is a Western blot of antibodies to GLRaV-3 that reacted to proteins produced by cDNA clones after IPTG induction in *E. coli*. Similar banding patterns were observed whether a polyclonal (panel A) or a monoclonal antibody (panel B) was used. Lane 1 shows clone pCP10-1; lane 2, pCP5; lane 3, pCP8-4; and lane 4, the native coat protein from GLRaV-3 infected tissue. Lane M is a prestained protein molecular weight marker.

Figure 9 shows the cDNA clones containing the coding region for the coat protein of the NY1 isolate of GLRaV-3. Three clones (pCP8-4, pCP5, pCP10-1) were identified by immunoscreening of a cDNA library prepared in lambda ZAP II. Two other clones were aligned after plaque hybridization and nucleotide sequencing. An ORF

encoding the coat protein is shown by an arrow in an open rectangle.

Figure 10 shows the nucleotide and amino acid sequences of the coat protein gene of grapevine leafroll associated closterovirus-3, isolate NY1. Nucleotide sequencing was conducted by the procedure described in Example 1. The translated amino acid sequence is shown below the nucleotide sequence.

Figure 11 compares the alignment of the coat protein of GLRaV-3 with respect to BYV, CTV, and LIYV. Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids, and lowercase letters indicate at least three identical or functionally similar amino acids. The three conserved amino acid residues (S, R, and D) identified in all filamentous plant virus coat proteins are in bold (Dolja et al., "Phylogeny of Capsid Proteins of Rod-shaped and Filamentous RNA Plant Viruses: Two Families with Distinct Patterns of Sequence and Probably Structure Conservation," Virology, 184:79-86 (1991)).

Figure 12 is a phylogenetic tree generated by the Clustal Method of MegAlign program in DNASTAR for the coat protein of GLRaV-3 with respect to that of other filamentous plant viruses. The coat protein of GLRaV-3 was incorporated into a previously described alignment (Dolja et al., "Molecular Biology and Evolution of Closteroviruses: Sophisticated Build-up of Large RNA Genomes," Annual Review of Phytopathology, 32:261-285 (1994) ("Dolja (1994)")) for comparison. The other virus sequences were obtained from current databases: apple chlorotic leafspot virus ("ACLSV"); apple stem grooving virus ("ASGV"); apple stem pitting virus ("ASPV"); barley yellow mosaic virus ("BaMV"); beet yellows closterovirus ("BYV"); diverged copies of BYV and CTV coat proteins ("BYV p24" and "CTV p27", respectively); citrus tristeza

virus ("CTV"); grapevine virus A ("GVA"); grapevine virus B ("GVB"); lily symptomless virus ("LSV"); lily virus X ("LVX"); narcissus mosaic virus ("NMV"); pepper mottle virus ("PeMV"); papaya mosaic virus ("PMV"); potato virus T ("PVT"); potato virus S ("PVS"); potato virus M ("PVM"); potato virus X ("PVX"); tobacco etch virus ("TEV"); tobacco vein mottle virus ("TVMV"); and white clover mosaic virus ("WcMV").

Figure 13 depicts an analysis of reverse transcription polymerase chain reaction ("RT-PCR") to detect GLRaV-3 in a partially purified virus preparation. The original sample concentration is equivalent to 50 mg/μl of phloem tissue (lane 1) which was diluted by 10-fold series as 10^{-1} (lane 2), 10^{-2} (lane 3), 10^{-3} (lane 4), 10^{-4} (lane 5), and 10^{-5} (lane 6), respectively. The expected size of 219 bp PCR product was clearly observed up to lane 4 which is equivalent to a detection limit of 10 μg of phloem tissue. Lane 7 was a healthy control. Lane 8 was dsRNA for positive control. Lanes 9-11 were also used for positive controls of purified viral RNA (lane 9), dsRNA (lane 10), and plasmid DNA (pC4) (lane 11) as templates, respectively. Lane M contains a molecular weight marker of *Hae* III digested fX 174 DNA.

Figure 14 shows the enzymatic inhibition in RT-PCR with proteinase K treated samples. By increasing amount of proteinase K treated sample in each 100 μl PCR reaction from 0.1 μl (lane 1) to 1 μl (lane 2) and to 10 μl (lane 3), an expected PCR product of 219 bp was readily observed in lane 1 (0.1 μl) and lane 2 (1 μl), but not in lane 3 (10 μl). The expected size of PCR product (219 bp) was also observed in GLRaV-3 dsRNA as positive control (lane 4), but not from proteinase K treated healthy grapevine tissue as negative control (lane 5). Lane M was the molecular weight standard of *Hae* III digested fX 174 DNA.

Figure 15 depicts a comparative analysis of Nested PCR with immuno-capture preparations on field collected samples. Using a polyclonal antibody to GLRaV-3 for immuno-capture, the expected PCR product of 648 bp was not consistently observable in the first round of PCR amplification with external primers over a range of samples (lanes 1-7, panel A). However, the expected PCR product of 219 bp amplified by internal primers was consistently observed over all seven samples (lanes 1-7, panel B). A similar inconsistency is also shown in a sample prepared by proteinase K-treated crude extract (compare panels A to B on lane 8). With dsRNA as template, the expected PCR products were readily observable in both reactions (compare panels A to B on lane 10). No such products were observed on a healthy sample (lane 9). Lane M was a molecular weight marker of Hae III digested fX 174 DNA.

Figure 16 depicts comparative studies on the sensitivity of Nested PCR with samples prepared by proteinase K-treated crude extract (panel A, PK Nested PCR) and by immuno-capture preparation (panel B, IC Nested PCR). Nested PCR was performed on samples with serial 10-fold dilutions of up to 10^{-6} in a proteinase K-treated (panel A) and 10^{-8} in an immuno-capture preparation (panel B). The expected PCR product of 219 bp was observable up to 10^{-5} in PK Nested PCR and over 10^{-8} (the highest dilution used in this test) in IC Nested PCR. A similar PCR product was also observed with dsRNA template but not from healthy grape tissues (H. CK). Lane M was a molecular weight marker of Hae III digested fX 174 DNA.

Figure 17 shows the partial genome organization of GLRaV-3 and the cDNA clones used to determine the nucleotide sequences. Numbered lines represent nucleotide coordinates in kilobases(kb).

Figures 18 A to W show the nucleotide sequence and partial genome organization of GLRaV-3.

Figure 19 depicts the proposed genome organization of the GLRaV-3 in comparison with three other closterovirus genomes, BYV, CTV, and LIYV (Dolja (1994)). Homologous proteins are shown by identical patterns. Papain-like proteinase ("P-PRO"); methyltransferase of type 1 ("MTR1"); RNA helicase of superfamily 1 ("HEL1"); RNA polymerase of supergroup 3 ("PLO3"); HSP70-related protein ("HSP70r"); and capsid protein forming filamentous virus particle ("CPf").

Figure 20 compares the amino acid sequence alignment of the helicase of GLRaV-3 with respect to BYV, CTV, and LIYV. Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids, lowercase letters indicate at least three identical or functionally similar amino acids. Six conserved motifs (I to VI) that are conserved among the Superfamily 1 helicase (Koonin et al., "Evolution and Taxonomy of Positive-strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences," Critical Reviews in Biochemistry and Molecular Biology, 28:375-430 (1993)) of the positive-strand RNA viruses are overlined.

Figure 21 is a phylogenetic tree showing the amino acid sequence relationship of helicase of alphaviruses. The helicase domain of GLRaV-3 (291 aa) from the present study is used. The other virus sequences were obtained from current databases (Swiss-Prot and GenBank, release 84.0). Apple chlorotic leafspot virus ("ACLSV"); broad bean mottle virus ("BbMV"); brome mosaic virus ("BMV"); beet yellow closterovirus ("BYV"); cowpea chlorotic mottle virus ("CcMV"); cucumber mosaic virus ("CMV"); fox mosaic virus ("FxMV"); lily symptomless virus ("LSV"); lily virus X ("LXV"); narcissus mosaic virus ("NMV"); pea early

browning virus ("PeBV"); papaya mosaic virus ("PMV"); poplar mosaic virus ("PopMV"); peanut stunt virus ("PSV"); potato virus S ("PVS"); potato virus M ("PVM"); potato virus X ("PVX"); strawberry mild yellow edge-associated virus ("Sm Yea V"); tomato aspermy virus ("TAV"); tobacco mosaic virus ("TMV"); tobacco rattle virus ("TRV"); and white clover mosaic virus ("WcMV").

Figure 22 compares the amino acid sequence alignment of the RNA dependent RNA polymerase (RdRp) of GLRaV-3 with respect to BYV, CTV, and LIYV. Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids, and lowercase letters indicate at least three identical or functionally similar amino acids. The motifs (I to VIII) that are conserved among the Supergroup 3 RNA polymerase of positive-strand RNA viruses are overlined.

Figure 23 shows the phylogenetic tree for the RNA dependent RNA polymerases (RdRp) of the alpha-like supergroup of positive strand RNA viruses. RdRp of GLRaV-3 was incorporated into a previously described alignment (Dolja (1994)) for comparison. The other virus sequences were obtained from current databases: Apple chlorotic leafspot virus ("ACLSV"); alfalfa mosaic virus ("AlMV"); apple stem grooving virus ("ASGV"); brome mosaic virus ("BMV"); beet necrotic yellow vein virus ("BNYVV"); beet yellow virus ("BYV"); barley stripe mosaic virus ("BSMV"); beet yellow stunt virus ("BYSV"); cucumber mosaic virus ("CMV"); citrus tristeza virus ("CTV"); hepatitis E virus ("HEV"); potato virus M ("PVM"); potato virus X ("PVX"); raspberry bushy dwarf virus ("RBDV"); shallot virus X ("SHVX"); Sinbis virus ("SNBV"); tobacco mosaic virus ("TMV"); tobacco rattle virus ("TRV"); and turnip yellow mosaic virus ("TYMV").

Figure 24 compares the alignment of the GLRaV-3 and LIYV nucleotide sequences (presented as DNA) in the

vicinity of the proposed frameshift, nt 4,099-4,165 in GLRaV-3 and nt 5,649-5,715 in LIYV. Identical nucleotides are typed in uppercase letters. LIYV +1 frameshift region (aAAG) and the corresponding GLRaV-3 (cACA) are bold and italic. The encoded C-terminus of HEL and N-terminus of RdRp are presented above (GLRaV-3) and below (LIYV) the nucleotide alignment. Repeat sequences are underlined.

Figure 25 compares the amino acid alignment of the small hydrophobic transmembrane protein of GLRaV-3 p5K with respect to BYV (p6K), CTV (p6K), and LIYV (p5K). Consensus amino acid residues are shown. Lowercase letters indicate at least three identical or functionally similar amino acids. The transmembrane domain that has been identified in several other closteroviruses, BYV, CTV, and LIYV (Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology, 208:511-520 (1995)), is overlined.

Figures 26 A to B present the amino acid sequence alignment of the HSP70-related protein of GLRaV-3 (p59K) with respect to BYV (p65K), CTV (p65K), and LIYV (p62K). The eight conserved motifs (A to H) of cellular HSP70 are overlined. Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids, and lowercase letters indicate at least three identical or functionally similar amino acids.

Figure 27 is a phylogenetic relationship for viral and cellular HSP70 proteins. HSP70-related protein of GLRaV-3 (p59) was incorporated into a previously described alignment (Dolja (1994)) for comparison. The sequences of BYV, CTV, and LIYV proteins were from Agranovsky et al., "Putative 65-kDa protein of Beet Yellows Closterovirus is a Homologue of HSP70 Heat Shock Proteins," Journal of General Virology, 217:603-610 (1991), Pappu et al., "Nucleotide Sequence and

Organization of Eight 3' Open Reading Frames of the Citrus Tristeza Closterovirus Genome," Virology, 199:35-46 (1994), and Klaassen et al., "Genome Structure and Phylogenetic Analysis of Lettuce Infectious Yellows Virus, a Whitefly-transmitted, Bipartite Closterovirus," Virology, 208:99-110 (1995), respectively. Only N-terminal half of beet yellow stunt virus HSP70-related protein (Karasev et al., "Screening of the Closterovirus Genome by Degenerate Primer-mediated Polymerase Chain Reaction," Journal of General Virology, 75:1415-1422 (1994)) is used. Other sequences were obtained from the Swiss-Prot database; their accession numbers are as follows: DNA1_BACSU, *Bacillus subtilis* (P13343); DNAK_ECOLI, *Escherichia coli* (P04475); HS70_CHICK (P08106); HS70_ONCMY, *Oncorhynchus mykiss* (P08108); HS70_PLACB, *Plasmodium cynomolgi* (Q05746); HS70_SCHMA, *Schistosoma mansoni* (P08418); HS70_XENLA, *Xenopus laevis* (P02827); HS71_DROME, *Drosophila melanogaster* (P02825); HS71_HUMAN (P08107); HS71_MOUSE (P17879); HS71_PIG (P34930); HS74_PARLI, *Paracentrotus lividus* (Q06248); HS74_TRYBB, *Trypanosoma brucei* (P11145); and ZMHSP702, maize gene for heat shock protein 70 exon 2 (X03697).

Figures 28 A to B compare the amino acid sequence alignment of the HSP90-related proteins of GLRaV-3 (p55K) with respect to BYV (p64K), CTV (p61K), and LIYV (p59K). Two domains, I and II, which have been identified on CTV (p61K) are overlined. Consensus amino acid residues are shown. Uppercase letters indicate identical amino acids; lowercase letters indicate at least three identical or functionally similar amino acids.

Figures 29 A to B show a nucleotide sequence fragment containing the 43 kDa open reading frame that was used to engineer a plant expression cassette, pBI525GLRaV-3hsp90. This sequence fragment (from

nucleotides 9,404 to 10,503 of the partial GLRaV-3 genome sequence, Figure 18) was later proven to be located in the 3' portion of GLRaV-3 HSP90-related gene. Nucleotides in the lower case were designed to facilitate engineering by addition of NcoI restriction sites.

Figure 30 is a diagram summarizing the strategies employed in the construction of the plant transformation vector pBin19GLRaV-3hsp90-12-3. A plant expression cassette, in the Hind III-EcoR I fragment containing CaMV 35S-35S promoters-AMV 5' untranslated sequence-43K ORF-Nos 3' untranslated region, was excised from pBI525GLRaV-3hsp90 and cloned into the similar restriction enzyme treated plant transformation vector pBin19. The resulting clone, pBin19GLRaV-3hsp90-12-3, is shown. Locations of important genetic elements within the binary plasmid are indicated: BR, right border; BL, left border; Nos-NPT II, plant expressible neomycin phosphotransferase gene; Lac-LAC Z, plant expressible Lac Z gene; and Bacterial Kan, bacterial kanamycin resistant gene.

Figure 31 presents an analysis of transgenic tobacco plants with PCR. Using primers flanking the 43K ORF, the proper size of PCR product (1.2 kb) was readily observed from 14 of the 18 kanamycin resistant plants. Lane ck shows a healthy control of nontransformed tobacco. Lane M shows a *Mr* marker of λ *Hind* III and fx174 *Hae* III.

Figure 32 shows the *Agrobacterium*-binary vector pGA482G/cpGLRaV-3, which was constructed by cloning the HindIII fragment of pEPT8cpGLRaV-3 into a derivative of pGA482 and used for transformation via *Agrobacterium* or Biolistic approach.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to isolated DNA molecules encoding for the proteins or polypeptides of a grapevine leafroll virus. Applicants have sequenced a substantial portion of the grapevine leafroll virus genome within which are a plurality of open reading frames, each containing DNA molecules in accordance with the present invention. One such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus helicase and comprises the nucleotide sequence corresponding to SEQ. ID. No. 1 as follows:

GTGTCTACTT ACGCGAAGAG TGTGATGAAC GACAATTTCA ATATCCTTGA
GACCCTGGTA ACTTTGCCCA AGTCCTTTAT AGTCAAAGTA CCTGGTTCGG
TGCTGGTTAG CATAACCACT TCGGGCATTT CCGACAAACT TGAACTTCGG
GGCGCGTTCG ACGTTTCTAA AAAGAATTTC TCCAGGAGGT TACGTTCGAG
TCGTTTGCGC GTATTTTCTA GGGCTATTGT GGAGGATACG ATCAAGGTTA
TGAAGGGCAT GAAATCAGAG GATGGTAAAC CACTCCCTAT AGCCGAGGAT
TCCGTGTACG CGTTCATGAC AGGCAATATG TCAAACGTTC ATTGCACTAG
GGCTGGTTTG CTCGGGGGCT CAAAGGCTTG CGCGGCTTCT TTAGCTGTGA
AGGGTGCAGC TTCACGCGCT ACTGGAACAA AACTCTTTTC AGGTCTCACA
TCCTTTCTTT CCGCCGGTGG TCTGTTTTAC GATGAAGGCT TGACGCCCGG
AGAGAGGCTT GATGCACTAA CGCGCCGTGA ACATGCTGTG AATTCACCTG
TAGGCCTCTT AGAACCTGGA GCTTCGGTTG CGAAGCGGGT CGTTTCCGGA
ACGAAAGCTT TTCTGTCAGA ATTGTCATTG GAGGACTTCA CCACTTTCGT
CATAAAAAAT AGGGTGCTTA TTGGTGTTTT TACTCTTTCC ATGGCTCTCA
CTCCGGTGGT CTGGAAGTAC AGAAGGAATA TCGCGCGAAC TGGCGTGGAT
GTTTTCCACC GTGCTCGTTC GGGTACCGCG GCCATCGGTT TACAATGTCT
TAGTGGAGGA AGGTCGTTAG CTGGTGACGC TGCTCGTGGC GCGTTAACAG
TGACTCGAGG AGGGCTATCT TCGGCGGTTG CGGTGACCAG AAATACAGTG
GCTAGGCGTC AGGTACCATT GGCGTTGCTT TCGTTTTCCA CGTCTTACGC
AGTCAGTGGT TGCACTTTGT TAGGTATTTG GGCTCATGCT CTCCCTAGGC
ATTTGATGTT CTTCTTTGGC CTAGGGACGC TCTTCGGGGT GAGTGCCAGT
ACCAATTCTT GGTCGCTTGG GGGCTATACG AACAGTCTGT TCACCGTACC

GGAATTAACT TGGGAAGGGA GGAGTTACAG ATCTTTATTG CCCCAAGCAG
CTTTAGGTAT TTCTCTCGTT GTGCGCGGGT TGTTAAGTGA AACTGTGCCA
CAACTAACGT ACGTACCGCC GATTGAAGGT CGGAATGTTT ATGATCAGGC
ACTAAATTTT TATCGCGACT TTGACTATGA CGATGGTGCA GGCCCATCCG
GGACGGCTGG TCAAAGCGAT CCTGGAACCA ATACTTCGGA TACTTCTTCG
GTTTTCTCTG ACGATGGTTT GCCCGCTAGT GGCGGTGGCT TCGACGCGCG
CGTTGAGGCA GGTCCCAGCC ATGCTGTTGA TGAATCACCA AGGGGTAGTG
TTGAGTTCGT CTACAGAGAA CGTGTAGATG AACATCCGGC GTGTGGTGAA
GCTGAAGTTG AAAAGGATCT AATAACACCA CTTGGTACAG CTGTCTTAGA
GTCGCCCCCC GTAGGTCCTG AAGCTGGGAG CGCGCCCAAC GTCGAGGACG
GTTGTCCGGA GGTTGAAGCT GAGAAATGTT CGGAGGTCAT CGTTGACGTT
CCTAGTTCAG AACCGCCGGT ACAAGAAGTC CTTGAATCAA CCAATGGTGT
CCAAGCTGCA AGAACTGAAG AGGTTGTGCA GGGCGACACA TGTGGAGCTG
GGGTAGCTAA ATCAGAAGTG AGTCAACGTG TGTTTCCTGC GCAAGTACCC
GCACATGAAG CTGGTCTTGA GGCATCTAGT GGCGCGGTCG TGGAGCCATT
GCAAGTTTCT GTGCCAGTAG CCGTAGAGAA AACTGTTTTA TCTGTCGAGA
AGGCGCGTGA GCTAAAGGCG GTAGATAAGG GCAAGGCGGT CGTGCACGCA
AAGGAAGTCA AGAATGTACC GGTTAAGACG TTACCACGAG GGGCTCTAAA
AATTAGTGAG GATACCGTTC GTAAGGAATT GTGCATGTTT AGAACGTGTT
CCTGCGGCGT GCAGTTGGAC GTGTACAATG AAGCGACCAT CGCCACTAGG
TTCTCAAATG CGTTTACCTT TGTCGATAGC TTGAAAGGGA GGAGTGCGGT
TTTCATCAGG GTGGCCTCGT GCCCTAGAGG ATATCTTAAC GGCAATTAAG
TACCCAAGCG TCTTCGACCA CTGTTTAGTG CAGAAGTACA AGATGGGTGG
AGGCGTACCA TTCCACGCTG ATGACGAGGA GTGCTATCCA TCAGATAACC
CTATCTTGAC GGTCAATCTC GTGGGGAAGG CAAACTTCTC GACTAAGTGC
AGGAAGGGTG GTAAGGTCAT GGTCATAAAC GTAGCTTCGG GTGACTATTT
TCTTATGCCT TGCGGTTTTC AAAGGACGCA CTTGCATTCA GTAAACTCCA
TCGACGAAGG GCGCATCAGT TTGACGTTCA GGGCAACTCG GCGCGTCTTT
GGTGTAGGCA GGATGTTGCA GTTAGCCGGC GGCGTGTCGG ATGAGAAGTC
ACCAGGTGTT CCAAACCAGC AACCACAGAG CCAAGGTGCT ACCAGAACAA
TCACACCAAA ATCGGGGGGC AAGGCTCTAT CTGAGGGAAG TGGTAGGGAA
GTCAAGGGGA GGTCGACATA CTCGATATGG TGCGAACAAG ATTACGTTAG
GAAGTGTGAG TGGCTCAGGG CTGATAATCC AGTGATGGCT CTTAAACCTG
GCTACACCCC AATGACATTT GAAGTGGTTA AAGCCGGGAC CTCTGAAGAT
GCCGTCGTGG AGTACTTGAA GTATCTGGCT ATAGGCATTG GGAGGACATA

CAGGGCGTTG CTTATGGCTA GAAATATTGC CGTCACTACC GCCGAAGGTG
TTCTGAAAGT ACCTAATCAA GTTTATGAAT CACTACCGGG CTTTCACGTT
TACAAGTCGG GCACAGATCT CATTTTTCAT TCAACACAAG ACGGCTTGCG
TGTGAGAGAC CTACCGTACG TATTCATAGC TGAGAAAGGT ATTTTTATCA
AGGGCAAAGA TGTCGACGCG GTAGTAGCTT TGGGCGACAA TCTGTCCGTA
TGTGATGATA TATTGGTTTT CCATGATGCT ATTAATTTGA TGGGTGCACT
GAAAGTTGCT CGATGTGGTA TGGTGGGTGA ATCATTTAAG TCGTTCGAAT
ACAAATGCTA TAATGCTCCC CCAGGTGGCG GTAAGACGAC GATGCTAGTG
GACGAATTTG TCAAGTCACC CAATAGCACG GCCACCATTA CGGCTAACGT
GGGAAGTTCT GAGGACATAA ATATGGCGGT GAAGAAGAGA GATCCGAATT
TGGAAGGTCT CAACAGTGCT ACCACAGTTA ACTCCAGGGT GGTTAACTTT
ATTGTCAGGG GAATGTATAA AAGGGTTTTG GTGGATGAGG TGTACATGAT
GCATCAAGGC TTACTACAAC TAGGCGTCTT CGCAACCGGC GCGTCGGAAG
GCCTCTTTTT TGGAGACATA AATCAGATAC CATTCATAAA CCGGGAGAAG
GTGTTTAGGA TGGATTGTGC TGTATTTGTT CCAAAGAAGG AAAGCGTTGT
ATACACTTCT AAATCATACA GGTGTCCGTT AGATGTTTGC TACTTGTTGT
CCTCAATGAC CGTAAGGGGA ACGGAAAAGT GTTACCCTGA AAAGGTCGTT
AGCGGTAAGG ACAAACCAGT AGTAAGATCG CTGTCCAAAA GGCCAATTGG
AACCACTGAT GACGTAGCTG AAATAAACGC TGACGTGTAC TTGTGCATGA
CCCAGTTGGA GAAGTCGGAT ATGAAGAGGT CGTTGAAGGG AAAAGGAAAA
GAAACACCAG TGATGACAGT GCATGAAGCA CAGGGAAAAA CATTCAGTGA
TGTGGTATTG TTTAGGACGA AGAAAGCCGA TGACTCCCTA TTCACTAAAC
AACCGCATAT ACTTGTTGGT TTGTCGAGAC ACACACGCTC ACTGGTTTAT
GCCGCTCTGA GCTCAGAGTT GGACGATAAG GTCGGCACAT ATATTAGCGA
CGCGTCGCCT CAATCAGTAT CCGACGCTTT GCTTCACACG TTCGCCCCGG
CTGGTTGCTT TCGAGGTATA TGA.

The helicase has an amino acid sequence corresponding to SEQ. ID. No. 2 as follows:

VSTYAKSVMN DNFNILETLV TLPKSFIVKV PGSVLVSITT SGISDKLELR
GAFDVSKKNF SRRLRSSRLR VFSRAIVEDT IKVMKGMKSE DGKPLPIAED
SVYAFMTGNM SNVHCTRAGL LGGSKACAAS LAVKGAASRA TGTKLFSGLT

SFLSAGGLFY DEGLTPGERL DALTRREHAV NSPVGLLEPG ASVAKRVVSG
TKAFLSELSL EDFTTFVIKN RVLIGVFTLS MALTPVVWKY RRNIARTGVD
VFHRARSGTA AIGLQCLSGG RSLAGDAARG ALTVTRGGLS SAVAVTRNTV
ARRQVPLALL SFSTSYAVSG CTLLGIWAHA LPRHLMFFFG LGTLFGVSAS
TNSWSLGGYT NSLFTVPELT WEGRSYRSLL PQAALGISLV VRGLLSETVP
QLTYVPPIEG RNVYDQALNF YRDFDYDDGA GPSGTAGQSD PGTNTSDTSS
VFSDDGLPAS GGGFDARVEA GPSHAVDESP RGSVEFVYRE RVDEHPACGE
AEVEKDLITP LGTAVLESPP VGPEAGSAPN VEDGCPEVEA EKCSEVIVDV
PSSEPPVQEV LESTNGVQAA RTEEVVQGDT CGAGVAKSEV SQRVFPAQVP
AHEAGLEASS GAVVEPLQVS VPVAVEKTVL SVEKARELKA VDKGKAVVHA
KEVKNVPVKT LPRGALKISE DTVRKELCMF RTCSCGVQLD VYNEATIATR
FSNAFTFVDS LKGRSAVFFS KLGEGYTYNG GSHVSSGWPR ALEDILTAIK
YPSVFDHCLV QKYKMGGGVP FHADDEECYP SDNPILTVNL VGKANFSTKC
RKGGKVMVIN VASGDYFLMP CGFQRTHLHS VNSIDEGRIS LTFRATRRVF
GVGRMLQLAG GVSDEKSPGV PNQQPQSQGA TRTITPKSGG KALSEGSGRE
VKGRSTYSIW CEQDYVRKCE WLRADNPVMA LKPGYTPMTF EVVKAGTSED
AVVEYLKYLA IGIGRTYRAL LMARNIAVTT AEGVLKVPNQ VYESLPGFHV
YKSGTDLIFH STQDGLRVRD LPYVFIAEKG IFIKGKDVDA VVALGDNLSV
CDDILVFHDA INLMGALKVA RCGMVGESFK SFEYKCYNAP PGGGKTTMLV
DEFVKSPNST ATITANVGSS EDINMAVKKR DPNLEGLNSA TTVNSRVVNF
IVRGMYKRVL VDEVYMMHQG LLQLGVFATG ASEGLFFGDI NQIPFINREK
VFRMDCAVFV PKKESVVYTS KSYRCPLDVC YLLSSMTVRG TEKCYPEKVV
SGKDKPVVRS LSKRPIGTTD DVAEINADVY LCMTQLEKSD MKRSLKGKGK
ETPVMTVHEA QGKTFSDVVL FRTKKADDSL FTKQPHILVG LSRHTRSLVY
AALSSELDDK VGTYISDASP QSVSDALLHT FAPAGCFRGI

and a molecular weight from about 146 to about 151 kDa, preferably about 148.5 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus RNA-dependent RNA polymerase and comprises the nucleotide sequence corresponding to SEQ. ID. No. 3 as follows:

ATGAATTTTG GACCGACCTT CGAAGGGGAG TTGGTACGGA AGATACCAAC
AAGTCATTTT GTAGCCGTGA ATGGGTTTCT CGAGGACTTA CTCGACGGTT
GTCCGGCTTT CGACTATGAC TTCTTTGAGG ATGATTTCGA AACTTCAGAT
CAGTCTTTCC TCATAGAAGA TGTGCGCATT TCTGAATCTT TTTCTCATTT
TGCGTCGAAA ATAGAGGATA GGTTTTACAG TTTTATTAGG TCTAGCGTAG
GTTTACCAAA GCGCAACACC TTGAAGTGTA ACCTCGTCAC GTTTGAAAAT
AGGAATTCCA ACGCCGATCG CGGTTGTAAC GTGGGTTGTG ACGACTCTGT
GGCGCATGAA CTGAAGGAGA TTTTCTTCGA GGAGGTCGTT AACAAAGCTC
GTTTAGCAGA GGTGACGGAA AGCCATTTGT CCAGCAACAC GATGTTGTTA
TCAGATTGGT TGGACAAAAG GGCACCTAAC GCTTACAAGT CTCTCAAGCG
GGCTTTAGGT TCGGTTGTCT TTCATCCGTC TATGTTGACG TCTTATACGC
TCATGGTGAA AGCAGACGTA AAACCCAAGT TGGACAATAC GCCATTGTCG
AAGTACGTAA CGGGGCAGAA TATAGTCTAC CACGATAGGT GCGTAACTGC
GCTTTTTTCT TGCATTTTTA CTGCGTGCGT AGAGCGCTTA AAATACGTAG
TGGACGAAAG GTGGCTCTTC TACCACGGGA TGGACACTGC GGAGTTGGCG
GCTGCATTGA GGAACAATTT GGGGGACATC CGGCAATACT ACACCTATGA
ACTGGATATC AGTAAGTACG ACAAATCTCA GAGTGCTCTC ATGAAGCAGG
TGGAGGAGTT GATACTCTTG ACACTTGGTG TTGATAGAGA AGTTTTGTCT
ACTTTCTTTT GTGGTGAGTA TGATAGCGTC GTGAGAACGA TGACGAAGGA
ATTGGTGTTG TCTGTCGGCT CTCAGAGGCG CAGTGGTGGT GCTAACACGT
GGTTGGGAAA TAGTTTAGTC TTGTGCACCT TGTTGTCCGT AGTACTTAGG
GGATTAGATT ATAGTTATAT TGTAGTTAGC GGTGATGATA GCCTTATATT
TAGTCGGCAG CCGTTGGATA TTGATACGTC GGTTCTGAGC GATAATTTTG
GTTTTGACGT AAAGATTTTT AACCAAGCTG CTCCATATTT TTGTTCTAAG
TTTTTAGTTC AAGTCGAGGA TAGTCTCTTT TTTGTTCCCG ATCCACTTAA
ACTCTTCGTT AAGTTTGGAG CTTCCAAAAC TTCAGATATC GACCTTTTAC
ATGAGATTTT TCAATCTTTC GTCGATCTTT CGAAGGGTTT CAATAGAGAG
GACGTCATCC AGGAATTAGC TAAGCTGGTG ACGCGGAAAT ATAAGCATTC
GGGATGGACC TACTCGGCTT TGTGTGTCTT GCACGTTTTA AGTGCAAATT
TTTCGCAGTT CTGTAGGTTA TATTACCACA ATAGCGTGAA TCTCGATGTG
CGCCCTATTC AGAGGACCGA GTCGCTTTCC TTGCTGGCCT TGAAGGCAAG
AATTTTAAGG TGGAAAGCTT CTCGTTTTGC CTTTTCGATA AAGAGGGGTT
AA.

The RNA-dependent RNA polymerase has an amino acid sequence corresponding to SEQ. ID. No. 4 as follows:

MNFGPTFEGE LVRKIPTSHF VAVNGFLEDL LDGCPAFDYD FFEDDFETSD
QSFLIEDVRI SESFSHFASK IEDRFYSFIR SSVGLPKRNT LKCNLVTFEN
RNSNADRGCN VGCDDSVAHE LKEIFFEEVV NKARLAEVTE SHLSSNTMLL
SDWLDKRAPN AYKSLKRALG SVVFHPSMLT SYTLMVKADV KPKLDNTPLS
KYVTGQNIVY HDRCVTALFS CIFTACVERL KYVVDERWLF YHGMDTAELA
AALRNNLGDI RQYYTYELDI SKYDKSQSAL MKQVEELILL TLGVDREVLS
TFFCGEYDSV VRTMTKELVL SVGSQRRSGG ANTWLGNSLV LCTLLSVVLR
GLDYSYIVVS GDDSLIFSRQ PLDIDTSVLS DNFGFDVKIF NQAAPYFCSK
FLVQVEDSLF FVPDPLKLFV KFGASKTSDI DLLHEIFQSF VDLSKGFNRE
DVIQELAKLV TRKYKHSGWT YSALCVLHVL SANFSQFCRL YYHNSVNLDV
RPIQRTESLS LLALKARILR WKASRFAFSI KRG

and a molecular weight from about 59 to about 63 kDa, preferably about 61 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus hsp70-related protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 5 as follows:

ATGGAAGTAG GTATAGATTT TGGAACCACT TTCAGCACAA TCTGCTTTTC
CCCATCTGGG GTCAGCGGTT GTACTCCTGT GGCCGGTAGT GTTTACGTTG
AAACCCAAAT TTTTATACCT GAAGGTAGCA GTACTTACTT AATTGGTAAA
GCTGCGGGGA AAGCTTATCG TGACGGTGTA GAGGGAAGGT TGTATGTTAA
CCCGAAAAGG TGGGCAGGTG TGACGAGGGA TAACGTCGAA CGCTACGTCG
AGAAATTAAA ACCTACATAC ACCGTGAAGA TAGACAGCGG AGGCGCCTTA
TTAATTGGAG GTTTAGGTTC CGGACCAGAC ACCTTATTGA GGGTCGTTGA
CGTAATATGT TTATTCTTGA GAGCCTTGAT ACTGGAGTGC GAAAGGTATA
CGTCTACGAC GGTTACAGCA GCTGTTGTAA CGGTACCGGC TGACTATAAC
TCCTTTAAAC GAAGCTTCGT TGTTGAGGCG CTAAAAGGTC TTGGTATACC
GGTTAGAGGT GTTGTTAACG AACCGACGGC CGCAGCCCTC TATTCCTTAG
CTAAGTCGCG AGTAGAAGAC CTATTATTAG CGGTTTTTGA TTTTGGGGGA

GGGACTTTCG ACGTCTCATT CGTTAAGAAG AAGGGAAATA TACTATGCGT
CATCTTTTCA GTGGGTGATA ATTTCTTGGG TGGTAGAGAT ATTGATAGAG
CTATCGTGGA AGTTATCAAA CAAAAGATCA AAGGAAAGGC GTCTGATGCC
AAGTTAGGGA TATTCGTATC CTCGATGAAG GAAGACTTGT CTAACAATAA
CGCTATAACG CAACACCTTA TCCCCGTAGA AGGGGGTGTG GAGGTTGTGG
ATTTGACTAG CGACGAACTG GACGCAATCG TTGCACCATT CAGCGCTAGG
GCTGTGGAAG TATTCAAAAC TGGTCTTGAC AACTTTTACC CAGACCCGGT
TATTGCCGTT ATGACTGGGG GGTCAAGTGC TCTAGTTAAG GTCAGGAGTG
ATGTGGCTAA TTTGCCGCAG ATATCTAAAG TCGTGTTCGA CAGTACCGAT
TTTAGATGTT CGGTGGCTTG TGGGGCTAAG GTTTACTGCG ATACTTTGGC
AGGTAATAGC GGACTGAGAC TGGTGGACAC TTTAACGAAT ACGCTAACGG
ACGAGGTAGT GGGTCTTCAG CCGGTGGTAA TTTTCCCGAA AGGTAGTCCA
ATACCCTGTT CATATACTCA TAGATACACA GTGGGTGGTG GAGATGTGGT
ATACGGTATA TTTGAAGGGG AGAATAACAG AGCTTTTCTA AATGAGCCGA
CGTTCCGGGG CGTATCGAAA CGTAGGGGAG ACCCAGTAGA GACCGACGTG
GCGCAGTTTA ATCTCTCCAC GGACGGAACG GTGTCTGTTA TCGTTAATGG
TGAGGAAGTA AAGAATGAAT ATCTGGTACC CGGGACAACA AACGTACTGG
ATTCATTGGT CTATAAATCT GGGAGAGAAG ATTTAGAGGC TAAGGCAATA
CCAGAGTACT TGACCACACT GAATATTTTG CACGATAAGG CTTTCACGAG
GAGAAACCTG GGTAACAAAG ATAAGGGGTT CTCGGATTTA AGGATAGAAG
AAAATTTTTT AAAATCCGCC GTAGATACAG ACACGATTTT GAATGGATAA.

The hsp70-related protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 6 as follows:

MEVGIDFGTT FSTICFSPSG VSGCTPVAGS VYVETQIFIP EGSSTYLIGK
AAGKAYRDGV EGRLYVNPKR WAGVTRDNVE RYVEKLKPTY TVKIDSGGAL
LIGGLGSGPD TLLRVVDVIC LFLRALILEC ERYTSTTVTA AVVTVPADYN
SFKRSFVVEA LKGLGIPVRG VVNEPTAAAL YSLAKSRVED LLLAVFDFGG
GTFDVSFVKK KGNILCVIFS VGDNFLGGRD IDRAIVEVIK QKIKGKASDA
KLGIFVSSMK EDLSNNNAIT QHLIPVEGGV EVVDLTSDEL DAIVAPFSAR
AVEVFKTGLD NFYPDPVIAV MTGGSSALVK VRSDVANLPQ ISKVVFDSTD
FRCSVACGAK VYCDTLAGNS GLRLVDTLTN TLTDEVVGLQ PVVIFPKGSP
IPCSYTHRYT VGGGDVVYGI FEGENNRAFL NEPTFRGVSK RRGDPVETDV

AQFNLSTDGT VSVIVNGEEV KNEYLVPGTT NVLDSLVYKS GREDLEAKAI
PEYLTTLNIL HDKAFTRRNL GNKDKGFSDL RIEENFLKSA VDTDTILNG

and a molecular weight from about 57 to about 61 kDa, preferably about 59 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus hsp90-related protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 7 as follows:

ATGGATAAAT ATATTTATGT AACGGGGATA TTAAACCCTA ACGAGGCTAG
AGACGAGGTA TTCTCGGTAG TGAATAAGGG ATATATTGGA CCGGGAGGGC
GCTCCTTTTC GAATCGTGGT AGTAAGTACA CCGTCGTCTG GGAAAACTCT
GCTGCGAGGA TTAGTGGATT TACGTCGACT TCGCAATCTA CGATAGATGC
TTTCGCGTAT TTCTTGTTGA AAGGCGGATT GACTACCACG CTCTCTAACC
CAATAAACTG TGAGAATTGG GTCAGGTCAT CTAAGGATTT AAGCGCGTTT
TTCAGGACCC TAATTAAAGG TAAGATTTAT GCATCGCGTT CTGTGGACAG
CAATCTTCCA AAGAAAGACA GGGATGACAT CATGGAAGCG AGTCGACGAC
TATCGCCATC GGACGCCGCC TTTTGCAGAG CAGTGTCGGT TCAGGTAGGG
AAGTATGTGG ACGTAACGCA GAATTTAGAA AGTACGATCG TGCCGTTAAG
AGTTATGGAA ATAAAGAAAA GACGAGGATC AGCACATGTT AGTTTACCGA
AGGTGGTATC CGCTTACGTA GATTTTTATA CGAACTTGCA GGAATTGCTG
TCGGATGAAG TAACTAGGGC CAGAACCGAT ACAGTTTCGG CATACGCTAC
CGACTCTATG GCTTTCTTAG TTAAGATGTT ACCCCTGACT GCTCGTGAGC
AGTGGTTAAA AGACGTGCTA GGATATCTGC TGGTACGGAG ACGACCAGCA
AATTTTTCCT ACGACGTAAG AGTAGCTTGG GTATATGACG TGATCGCTAC
GCTCAAGCTG GTCATAAGAT TGTTTTTCAA CAAGGACACA CCCGGGGGTA
TTAAAGACTT AAAACCGTGT GTGCCTATAG AGTCATTCGA CCCCTTTCAC
GAGCTTTCGT CCTATTTCTC TAGGTTAAGT TACGAGATGA CGACAGGTAA
AGGGGGAAAG ATATGCCCGG AGATCGCCGA GAAGTTGGTG CGCCGTCTAA
TGGAGGAAAA CTATAAGTTA AGATTGACCC CAGTGATGGC CTTAATAATT
ATACTGGTAT ACTACTCCAT TTACGGCACA AACGCTACCA GGATTAAAAG
ACGCCCGGAT TTCCTCAATG TGAGGATAAA GGGAAGAGTC GAGAAGGTTT
CGTTACGGGG GGTAGAAGAT CGTGCCTTTA GAATATCAGA AAAGCGCGGG

ATAAACGCTC AACGTGTATT ATGTAGGTAC TATAGCGATC TCACATGTCT
GGCTAGGCGA CATTACGGCA TTCGCAGGAA CAATTGGAAG ACGCTGAGTT
ATGTAGACGG GACGTTAGCG TATGACACGG CTGATTGTAT AACTTCTAAG
GTGAGAAATA CGATCAACAC CGCAGATCAC GCTAGCATTA TACACTATAT
CAAGACGAAC GAAAACCAGG TTACCGGAAC TACTCTACCA CACCAGCTTT
AA.

The hsp90-related protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 8 as follows:

MDKYIYVTGI LNPNEARDEV FSVVNKGYIG PGGRSFSNRG SKYTVVWENS
AARISGFTST SQSTIDAFAY FLLKGGLTTT LSNPINCENW VRSSKDLSAF
FRTLIKGKIY ASRSVDSNLP KKDRDDIMEA SRRLSPSDAA FCRAVSVQVG
KYVDVTQNLE STIVPLRVME IKKRRGSAHV SLPKVVSAYV DFYTNLQELL
SDEVTRARTD TVSAYATDSM AFLVKMLPLT AREQWLKDVL GYLLVRRRPA
NFSYDVRVAW VYDVIATLKL VIRLFFNKDT PGGIKDLKPC VPIESFDPFH
ELSSYFSRLS YEMTTGKGGK ICPEIAEKLV RRLMEENYKL RLTPVMALII
ILVYYSIYGT NATRIKRRPD FLNVRIKGRV EKVSLRGVED RAFRISEKRG
INAQRVLCRY YSDLTCLARR HYGIRRNNWK TLSYVDGTLA YDTADCITSK
VRNTINTADH ASIIHYIKTN ENQVTGTTLP HQL

and a molecular weight from about 53 to about 57 kDa, preferably about 55 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus coat protein or polypeptide. The DNA molecule comprises the nucleotide sequence corresponding to SEQ. ID. No. 9 as follows:

ATGGCATTTG AACTGAAATT AGGGCAGATA TATGAAGTCG TCCCCGAAAA
TAATTTGAGA GTTAGAGTGG GGGATGCGGC ACAAGGAAAA TTTAGTAAGG
CGAGTTTCTT AAAGTACGTT AAGGACGGGA CACAGGCGGA ATTAACGGGA
ATCGCCGTAG TGCCCGAAAA ATACGTATTC GCCACAGCAG CTTTGGCTAC
AGCGGCGCAG GAGCCACCTA GGCAGCCACC AGCGCAAGTG GCGGAACCAC

AGGAAACCGA TATAGGGGTA GTGCCGGAAT CTGAGACTCT CACACCAAAT
AAGTTGGTTT TCGAGAAAGA TCCAGACAAG TTCTTGAAGA CTATGGGCAA
GGGAATAGCT TTGGACTTGG CGGGAGTTAC CCACAAACCG AAAGTTATTA
ACGAGCCAGG GAAAGTATCA GTAGAGGTGG CAATGAAGAT TAATGCCGCA
TTGATGGAGC TGTGTAAGAA GGTTATGGGC GCCGATGACG CAGCAACTAA
GACAGAATTC TTCTTGTACG TGATGCAGAT TGCTTGCACG TTCTTTACAT
CGTCTTCGAC GGAGTTCAAA GAGTTTGACT ACATAGAAAC CGATGATGGA
AAGAAGATAT ATGCGGTGTG GGTATATGAT TGCATTAAAC AAGCTGCTGC
TTCGACGGGT TATGAAAACC CGGTAAGGCA GTATCTAGCG TACTTCACAC
CAACCTTCAT CACGGCGACC CTGAATGGTA AACTAGTGAT GAACGAGAAG
GTTATGGCAC AGCATGGAGT ACCACCGAAA TTCTTTCCGT ACACGATAGA
CTGCGTTCGT CCGACGTACG ATCTGTTCAA CAACGACGCA ATATTAGCAT
GGAATTTAGC TAGACAGCAG GCGTTTAGAA ACAAGACGGT AACGGCCGAT
AACACCTTAC ACAACGTCTT CCAACTATTG CAAAAGAAGT AG.

The coat protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 10 as follows:

MAFELKLGQI YEVVPENNLR VRVGDAAQGK FSKASFLKYV KDGTQAELTG
IAVVPEKYVF ATAALATAAQ EPPRQPPAQV AEPQETDIGV VPESETLTPN
KLVFEKDPDK FLKTMGKGIA LDLAGVTHKP KVINEPGKVS VEVAMKINAA
LMELCKKVMG ADDAATKTEF FLYVMQIACT FFTSSSTEFK EFDYIETDDG
KKIYAVWVYD CIKQAAASTG YENPVRQYLA YFTPTFITAT LNGKLVMNEK
VMAQHGVPPK FFPYTIDCVR PTYDLFNNDA ILAWNLARQQ AFRNKTVTAD
NTLHNVFQLL QKK

and a molecular weight from about 33 to about 43 kDa, preferably about 35 kDa.

Alternatively, the DNA molecule of the present invention can constitute an open reading frame which codes for a first undefined protein or polypeptide. This DNA molecule comprises the nucleotide sequence corresponding to SEQ. ID. No. 11 as follows:

ATGTACAGTA GAGGGTCTTT CTTTAAGTCT CGGGTTACCC TTCCTACTCT

TGTCGGAGCA TACATGTGGG AGTTTGAACT CCCGTATCTT ACGGACAAGA GACACATCAG CTATAGCGCG CCAAGTGTCG CGACTTTTAG CCTTGTGTCG AGGTAG.

The first undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 12 as follows:

MYSRGSFFKS RVTLPTLVGA YMWEFELPYL TDKRHISYSA PSVATFSLVS R

and a molecular weight from about 5 to about 7 kDa, preferably about 6 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a second undefined grapevine leafroll virus protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 13 as follows:

ATGGATGATT TTAAACAGGC AATACTGTTG CTAGTAGTCG ATTTTGTCTT CGTGATAATT CTGCTGCTGG TTCTTACGTT CGTCGTCCCG AGGTTACAGC AAAGCTCCAC CATTAATACA GGTCTTAGGA CAGTGTGA.

The second undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 14 as follows:

MDDFKQAILL LVVDFVFVII LLLVLTFVVP RLQQSSTINT GLRTV

and a molecular weight from about 4 to about 6 kDa, preferably about 5 kDa.

Another such DNA molecule constitutes an open reading frame which codes for a grapevine leafroll virus coat protein or polypeptide repeat and comprises

the nucleotide sequence corresponding to SEQ. ID. No. 15 as follows:—

ATGGGAGCTT ATACACATGT AGACTTTCAT GAGTCGCGGT TGCTGAAAGA
CAAACAAGAC TATCTTTCTT TCAAGTCAGC GGATGAAGCT CCTCCTGATC
CTCCCGGATA CGTTCGCCCA GATAGTTATG TGAGGGCTTA TTTGATACAA
AGAGCAGACT TTCCCAATAC TCAAAGCTTA TCAGTTACGT TATCGATAGC
CAGTAATAAG TTAGCTTCAG GTCTTATGGG AAGCGACGCA GTATCATCGT
CGTTTATGCT GATGAACGAC GTGGGAGATT ACTTCGAGTG CGGCGTGTGT
CACAACAAAC CCTACTTAGG ACGGGAAGTT ATCTTCTGTA GGAAATACAT
AGGTGGGAGA GGAGTGGAGA TCACCACTGG TAAGAACTAC ACGTCGAACA
ATTGGAACGA GGCGTCGTAC GTAATACAAG TGAACGTAGT CGATGGGTTA
GCACAGACCA CTGTTAATTC TACTTATACG CAAACGGACG TTAGTGGTCT
ACCCAAAAAT TGGACGCGTA TCTACAAAAT AACAAAGATA GTGTCCGTAG
ATCAGAACCT CTACCCTGGT TGTTTCTCAG ACTCGAAACT GGGTGTAATG
CGTATAAGGT CACTGTTAGT TTCCCCAGTG CGCATCTTCT TTAGGGATAT
CTTATTGAAA CCTTTGAAGA AATCGTTCAA CGCAAGAATC GAGGATGTGC
TGAATATTGA CGACACGTCG TTGTTAGTAC CGAGTCCTGT CGTACCAGAG
TCTACGGGAG GTGTAGGTCC ATCAGAGCAG CTGGATGTAG TGGCTTTAAC
GTCCGACGTA ACGGAATTGA TCAACACTAG GGGGCAAGGT AAGATATGTT
TTCCAGACTC AGTGTTATCG ATCAATGAAG CGGATATCTA CGATGAGCGG
TATTTGCCGA TAACGGAAGC TCTACAGATA AACGCAAGAC TACGCAGACT
CGTTCTTTCG AAAGGCGGGA GTCAAACACC ACGAGATATG GGGAATATGA
TAGTGGCCAT GATACAACTT TTCGTACTCT ACTCTACTGT AAAGAATATA
AGCGTCAAAG ACGGGTATAG GGTGGAGACC GAATTAGGTC AAAAGAGAGT
CTACTTAAGT TATTCGGAAG TAAGGGAAGC TATATTAGGA GGGAAATACG
GTGCGTCTCC AACCAACACT GTGCGATCCT TCATGAGGTA TTTTGCTCAC
ACCACTATTA CTCTACTTAT AGAGAAGAAA ATTCAGCCAG CGTGTACTGC
CCTAGCTAAG CACGGCGTCC CGAAGAGGTT CACTCCGTAC TGCTTCGACT
TCGCACTACT GGATAACAGA TATTACCCGG CGGACGTGTT GAAGGCTAAC
GCAATGGCTT GCGCTATAGC GATTAAATCA GCTAATTTAA GGCGTAAAGG
TTCGGAGACG TATAACATCT TAGAAAGCAT TTGA.

The grapevine leafroll virus coat protein or polypeptide repeat has an amino acid sequence corresponding to SEQ. ID. No. 16 as follows:

MGAYTHVDFH ESRLLKDKQD YLSFKSADEA PPDPPGYVRP DSYVRAYLIQ
RADFPNTQSL SVTLSIASNK LASGLMGSDA VSSSFMLMND VGDYFECGVC
HNKPYLGREV IFCRKYIGGR GVEITTGKNY TSNNWNEASY VIQVNVVDGL
AQTTVNSTYT QTDVSGLPKN WTRIYKITKI VSVDQNLYPG CFSDSKLGVM
RIRSLLVSPV RIFFRDILLK PLKKSFNARI EDVLNIDDTS LLVPSPVVPE
STGGVGPSEQ LDVVALTSDV TELINTRGQG KICFPDSVLS INEADIYDER
YLPITEALQI NARLRRLVLS KGGSQTPRDM GNMIVAMIQL FVLYSTVKNI
SVKDGYRVET ELGQKRVYLS YSEVREAILG GKYGASPTNT VRSFMRYFAH
TTITLLIEKK IQPACTALAK HGVPKRFTPY CFDFALLDNR YYPADVLKAN
AMACAIAIKS ANLRRKGSET YNILESI

and a molecular weight from about 51 to about 55 kDa, preferably about 53 kDa.

Yet another such DNA molecule constitutes an open reading frame which codes for a third undefined grapevine leafroll virus protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 17 as follows:

ATGGAATTCA GACCAGTTTT AATTACAGTT CGCCGTGATC CCGGCGTAAA
CACTGGTAGT TTGAAAGTGA TAGCTTATGA CTTACACTAC GACAATATAT
TCGATAACTG CGCGGTAAAG TCGTTTCGAG ACACCGACAC TGGATTCACT
GTTATGAAAG AATACTCGAC GAATTCAGCG TTCATACTAA GTCCTTATAA
ACTGTTTTCC GCGGTCTTTA ATAAGGAAGG TGAGATGATA AGTAACGATG
TAGGATCGAG TTTCAGGGTT TACAATATCT TTTCGCAAAT GTGTAAAGAT
ATCAACGAGA TCAGCGAGAT ACAACGCGCC GGTTACCTAG AAACATATTT
AGGAGACGGG CAGGCTGACA CTGATATATT TTTTGATGTC TTAACCAACA
ACAAAGCAAA GGTAAGGTGG TTAGTTAATA AAGACCATAG CGCGTGGTGT
GGGATATTGA ATGATTTGAA GTGGGAAGAG AGCAACAAGG AGAAATTTAA
GGGGAGAGAC ATACTAGATA CTTACGTTTT ATCGTCTGAT TATCCAGGGT
TTAAATGA.

The third undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 18 as follows:

MEFRPVLITV RRDPGVNTGS LKVIAYDLHY DNIFDNCAVK SFRDTDTGFT VMKEYSTNSA FILSPYKLFS AVFNKEGEMI SNDVGSSFRV YNIFSQMCKD INEISEIQRA GYLETYLGDG QADTDIFFDV LTNNKAKVRW LVNKDHSAWC GILNDLKWEE SNKEKFKGRD ILDTYVLSSD YPGFK

and a molecular weight from about 33 to about 39 kDa, preferably about 36 kDa.

Yet another such DNA molecule constitutes an open reading frame which codes for a fourth undefined grapevine leafroll virus protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 19 as follows:

ATGAAGTTGC TTTCGCTCCG CTATCTTATC TTAAGGTTGT CAAAGTCGCT TAGAACGAAC GATCACTTGG TTTTAATACT TATAAAGGAG GCGCTTATAA ACTATTACAA CGCCTCTTTC ACCGATGAGG GTGCCGTATT AAGAGACTCT CGCGAAAGTA TAGAGAATTT TCTCGTAGCC AGGTGCGGTT CGCAAAATTC CTGCCGAGTC ATGAAGGCTT TGATCACTAA CACAGTCTGT AAGATGTCGA TAGAAACAGC CAGAAGTTTT ATCGGAGACT TAATACTCGT CGCCGACTCC TCTGTTTCAG CGTTGGAAGA AGCGAAATCA ATTAAAGATA ATTTCCGCTT AAGAAAAAGG AGAGGCAAGT ATTATTATAG TGGTGATTGT GGATCCGACG TTGCGAAAGT TAAGTATATT TTGTCTGGGG AGAATCGAGG ATTGGGGTGC GTAGATTCCT TGAAGCTAGT TTGCGTAGGT AGACAAGGAG GTGGAAACGT ACTACAGCAC CTACTAATCT CATCTCTGGG TTAA.

The fourth undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 20 as follows:

MKLLSLRYLI LRLSKSLRTN DHLVLILIKE ALINYYNASF TDEGAVLRDS RESIENFLVA RCGSQNSCRV MKALITNTVC KMSIETARSF IGDLILVADS

SVSALEEAKS IKDNFRLRKR RGKYYYSGDC GSDVAKVKYI LSGENRGLGC
VDSLKLVCVG RQGGGNVLQH LLISSLG

and a molecular weight from about 17 to about 23 kDa, preferably about 20 kDa.

Yet another such DNA molecule constitutes an open reading frame which codes for a fifth undefined grapevine leafroll virus protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 21 as follows:

ATGGACCTAT CGTTTATTAT TGTGCAGATC CTTTCCGCCT CGTACAATAA
TGACGTGACA GCACTTTACA CTTTGATTAA CGCGTATAAT AGCGTTGATG
ATACGACGCG CTGGGCAGCG ATAAACGATC CGCAAGCTGA GGTTAACGTC
GTGAAGGCTT ACGTAGCTAC TACAGCGACG ACTGAGCTGC ATAGAACAAT
TCTCATTGAC AGTATAGACT CCGCCTTCGC TTATGACCAA GTGGGGTGTT
TGGTGGGCAT AGCTAGAGGT TTGCTTAGAC ATTCGGAAGA TGTTCTGGAG
GTCATCAAGT CGATGGAGTT ATTCGAAGTG TGTCGTGGAA AGAGGGGAAG
CAAAAGATAT CTTGGATACT TAAGTGATCA ATGCACTAAC AAATACATGA
TGCTAACTCA GGCCGGACTG GCCGCAGTTG AAGGAGCAGA CATACTACGA
ACGAATCATC TAGTCAGTGG TAATAAGTTC TCTCCAAATT TCGGGATCGC
TAGGATGTTG CTCTTGACGC TTTGTTGCGG AGCACTATAA.

The fifth undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 22 as follows:

MDLSFIIVQI LSASYNNDVT ALYTLINAYN SVDDTTRWAA INDPQAEVNV
VKAYVATTAT TELHRTILID SIDSAFAYDQ VGCLVGIARG LLRHSEDVLE
VIKSMELFEV CRGKRGSKRY LGYLSDQCTN KYMMLTQAGL AAVEGADILR
TNHLVSGNKF SPNFGIARML LLTLCCGAL

and a molecular weight from about 17 to about 23 kDa, preferably about 20 kDa.

Yet another such DNA molecule constitutes an open reading frame which codes for a sixth undefined grapevine leafroll virus protein or polypeptide and comprises the nucleotide sequence corresponding to SEQ. ID. No. 23 as follows:

ATGAGGCACT TAGAAAAACC CATCAGAGTA GCGGTACACT ATTGCGTCGT
GCGAAGTGAC GTTTGTGACG GGTGGGATGT ATTTATAGGC GTAACGTTAA
TCGGTATGTT TATTAGTTAC TATTTATATG CTCTAATTAG CATATGTAGA
AAAGGAGAAG GTTTAACAAC CAGTAATGGG TAA.

The sixth undefined protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 24 as follows:

MRHLEKPIRV AVHYCVVRSD VCDGWDVFIG VTLIGMFISY YLYALISICR
KGEGLTTSNG

and a molecular weight from about 5 to about 9 kDa, preferably about 7 kDa.

Also encompassed by the present invention are fragments of the DNA molecules of the present invention. Suitable fragments capable of imparting grapevine leafroll resistance to grape plants are constructed by using appropriate restriction sites, revealed by inspection of the DNA molecule's sequence, to: (i) insert an interposon (Felley et al., "Interposon Mutagenesis of Soil and Water Bacteria: a Family of DNA Fragments Designed for in vitro Insertion Mutagenesis of Gram-negative Bacteria," Gene, 52:147-15 (1987), which is hereby incorporated by reference) such that truncated forms of the grapevine leafroll virus coat polypeptide or protein, that lack various amounts of the C-terminus, can be produced or (ii) delete various internal portions of the protein.

Alternatively, the sequence can be used to amplify any portion of the coding region, such that it can be cloned into a vector supplying both transcription and translation start signals.

Variants may also (or alternatively) be modified by, for example, the deletion or addition of nucleotides that have minimal influence on the properties, secondary structure and hydropathic nature of the encoded polypeptide. For example, the nucleotides encoding a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The nucleotide sequence may also be altered so that the encoded polypeptide is conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

The protein or polypeptide of the present invention is preferably produced in purified form (preferably, at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is isolated by lysing and sonication. After washing, the lysate pellet is resuspended in buffer containing Tris-HCl. During dialysis, a precipitate forms from this protein solution. The solution is centrifuged, and the pellet is washed and resuspended in the buffer containing Tris-HCl. Proteins are resolved by electrophoresis through an SDS 12% polyacrylamide gel.

The DNA molecule encoding the grapevine leafroll virus protein or polypeptide of the present invention can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is

heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology, vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard

cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria or transformed via particle bombardment (i.e. biolistics). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA ("mRNA") translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno ("SD") sequence about 7-9 bases 5′ to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecules encoding the various grapevine leafroll virus proteins or polypeptides, as described above, have been cloned into an expression system, they are ready to be incorporated into a host cell. Such incorporation can be carried

out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention also relates to RNA molecules which encode the various grapevine leafroll virus proteins or polypeptides described above. The transcripts can be synthesized using the host cells of the present invention by any of the conventional techniques. The mRNA can be translated either *in vitro* or *in vivo*. Cell-free systems typically include wheat-germ or reticulocyte extracts. *In vivo* translation can be effected, for example, by microinjection into frog oocytes.

One aspect of the present invention involves using one or more of the above DNA molecules encoding the various proteins or polypeptides of a grapevine leafroll virus to transform grape plants in order to impart grapevine leafroll resistance to the plants. The mechanism by which resistance is imparted in not known. In one hypothetical mechanism, the transformed plant can express the coat protein or polypeptide, and, when the transformed plant is inoculated by a grapevine leafroll virus, such as GLRaV-1, GLRaV-2, GLRav-3, GLRaV-4, GLRaV-5, or GLRaV-6, or combinations of these, the expressed coat protein or polypeptide surrounds the virus, thereby preventing translation of the viral DNA.

In this aspect of the present invention the subject DNA molecule incorporated in the plant can be constitutively expressed. Alternatively, expression can be regulated by a promoter which is activated by the presence of grapevine leafroll virus. Suitable promoters for these purposes include those from genes

expressed in response to grapevine leafroll virus infiltration.

The isolated DNA molecules of the present invention can be utilized to impart grapevine leafroll resistance for a wide variety of grapevine plants. The DNA molecules are particularly well suited to imparting resistance to *Vitis* scion or rootstock cultivars. Scion cultivars which can be protected include those commonly referred to as Table on Raisin Grapes, such as Alden, Almeria, Anab-E-Shahi, Autumn Black, Beauty Seedless, Black Corinth, Black Damascus, Black Malvoisie, Black Prince, Blackrose, Bronx Seedless, Burgrave, Calmeria, Campbell Early, Canner, Cardinal, Catawba, Christmas, Concord, Dattier, Delight, Diamond, Dizmar, Duchess, Early Muscat, Emerald Seedless, Emperor, Exotic, Ferdinand de Lesseps, Fiesta, Flame seedless, Flame Tokay, Gasconade, Gold, Himrod, Hunisa, Hussiene, Isabella, Italia, July Muscat, Khandahar, Katta, Kourgane, Kishmishi, Loose Perlette, Malaga, Monukka, Muscat of Alexandria, Muscat Flame, Muscat Hamburg, New York Muscat, Niabell, Niagara, Olivette blanche, Ontario, Pierce, Queen, Red Malaga, Ribier, Rish Baba, Romulus, Ruby Seedless, Schuyler, Seneca, Suavis (IP 365), Thompson seedless, and Thomuscat. They also include those used in wine production, such as Aleatico, Alicante Bouschet, Aligote, Alvarelhao, Aramon, Baco blanc (22A), Burger, Cabernet franc, Cabernet, Sauvignon, Calzin, Carignane, Charbono, Chardonnay, Chasselas dore, Chenin blanc, Clairette blanche, Early Burgundy, Emerald Riesling, Feher Szagos, Fernao Pires, Flora, French Colombard, Fresia, Furmint, Gamay, Gewurztraminer, Grand noir, Gray Riesling, Green Hungarian, Green Veltliner, Grenache, Grillo, Helena, Inzolia, Lagrein, Lambrusco de Salamino, Malbec, Malvasia bianca, Mataro, Melon,

Merlot, Meunier, Mission, Montua de Pilas, Muscadelle du Bordelais, Muscat blanc, Muscat Ottonel, Muscat Saint-Vallier, Nebbiolo, Nebbiolo fino, Nebbiolo Lampia, Orange Muscat, Palomino, Pedro Ximenes, Petit Bouschet, Petite Sirah, Peverella, Pinot noir, Pinot Saint-George, Primitivo di Gioa, Red Veltliner, Refosco, Rkatsiteli, Royalty, Rubired, Ruby Cabernet, Saint-Emilion, Saint Macaire, Salvador, Sangiovese, Sauvignon blanc, Sauvignon gris, Sauvignon vert, Scarlet, Seibel 5279, Seibel 9110, Seibel 13053, Semillon, Servant, Shiraz, Souzao, Sultana Crimson, Sylvaner, Tannat, Teroldico, Tinta Madeira, Tinto cao, Touriga, Traminer, Trebbiano Toscano, Trousseau, Valdepenas, Viognier, Walschriesling, White Riesling, and Zinfandel. Rootstock cultivars which can be protected include Couderc 1202, Couderc 1613, Couderc 1616, Couderc 3309, Dog Ridge, Foex 33 EM, Freedom, Ganzin 1 (A x R #1), Harmony, Kober 5BB, LN33, Millardet & de Grasset 41B, Millardet & de Grasset 420A, Millardet & de Grasset 101-14, Oppenheim 4 (SO4), Paulsen 775, Paulsen 1045, Paulsen 1103, Richter 99, Richter 110, Riparia Gloire, Ruggeri 225, Saint-George, Salt Creek, Teleki 5A, Vitis rupestris Constantia, *Vitis californica*, and *Vitis girdiana*.

There exists an extensive similarity in the hsp70-related sequence regions of GLRaV-3 and other closteroviruses, such as tristeza virus. Consequently, the GLRaV-3 hsp70-related gene can also be used to produce transgenic cultivars other than grape, such as citrus, which are resistant to closteroviruses other than grapevine leafroll, such as tristeza virus. These include cultivars of lemon, lime, orange, grapefruit, pineapple, tangerine, and the like, such as Joppa, Maltaise Ovale, Parson (Parson Brown), Pera, Pineapple, Queen, Shamouti, Valencia, Tenerife, Imperial

Doblefina, Washington Sanguine, Moro, Sanguinello Moscato, Spanish Sanguinelli, Tarocco, Atwood, Australian, Bahia, Baiana, Cram, Dalmau, Eddy, Fisher, Frost Washington, Gillette, LengNavelina, Washington, Satsuma Mandarin, Dancy, Robinson, Ponkan, Duncan, Marsh, Pink Marsh, Ruby Red, Red Seedless, Smooth Seville, Orlando Tangelo, Eureka, Lisbon, Meyer Lemon', Rough Lemon, Sour Orange, Persian Lime, West Indian Lime, Bearss, Sweet Lime, Troyer Citrange, and Citrus trifoliata.

Plant tissue suitable for transformation include leaf tissue, root tissue, meristems, zygotic and somatic embryos, and anthers. It is particularly preferred to utilize embryos obtained from anther cultures.

The expression system of the present invention can be used to transform virtually any plant tissue under suitable conditions. Tissue cells transformed in accordance with the present invention can be grown *in vitro* in a suitable medium to impart grapevine leafroll virus resistance. Transformed cells can be regenerated into whole plants such that the protein or polypeptide imparts resistance to grapevine leafroll virus in the intact transgenic plants. In either case, the plant cells transformed with the recombinant DNA expression system of the present invention are grown and caused to express that DNA molecule to produce one of the above-described grapevine leafroll virus proteins or polypeptides and, thus, to impart grapevine leafroll resistance.

One technique of transforming plants with the DNA molecules in accordance with the present invention is by contacting the tissue of such plants with an inoculum of a bacteria transformed with a vector comprising a gene in accordance with the present

invention which imparts grapevine leafroll resistance. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains C58, LBA4404, or EHA105) is particularly useful due to its well-known ability to transform plants.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., and in Emerschad et al., "Somatic Embryogenesis and Plant Development from Immature Zygotic Embryos of Seedless Grapes (*Vitis vinifera*)," Plant Cell Reports, 14:6-12 (1995) ("Emerschad (1995)"), which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle.

Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Once a grape plant tissue is transformed in accordance with the present invention, it is regenerated to form a transgenic grape plant. Generally, regeneration is accomplished by culturing transformed tissue on medium containing the appropriate growth regulators and nutrients to allow for the initiation of shoot meristems. Appropriate antibiotics are added to the regeneration medium to inhibit the growth of *Agrobacterium* and to select for the development of transformed cells. Following shoot initiation, shoots are allowed to develop tissue culture and are screened for marker gene activity.

The DNA molecules of the present invention can be made capable of transcription to a messenger RNA, which, although encoding for a grapevine leafroll virus protein or polypeptide, does not translate to the protein. This is known as RNA-mediated resistance. When a *Vitis* scion or rootstock cultivar is transformed with such a DNA molecule, the DNA molecule can be transcribed under conditions effective to maintain the messenger RNA in the plant cell at low level density readings. Density readings of between 15 and 50 using a Hewlet ScanJet and Image Analysis Program are preferred.

The grapevine leafroll virus protein or polypeptide can also be used to raise antibodies or binding portions thereof or probes. The antibodies can be monoclonal or polyclonal.

Monoclonal antibody production may be effected by techniques which are well-known in the art. Basically, the process involves first obtaining immune cells (lymphocytes) from the spleen of a mammal (e.g.,

mouse) which has been previously immunized with the antigen of interest either *in vivo* or *in vitro*. The antibody-secreting lymphocytes are then fused with (mouse) myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured, and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler and Milstein, Nature, 256:495 (1975), which is hereby incorporated by reference.

Mammalian lymphocytes are immunized by *in vivo* immunization of the animal (e.g., a mouse) with the protein or polypeptide of the present invention. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Following the last antigen boost, the animals are sacrificed and spleen cells removed.

Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents. (See Milstein and Kohler, Eur. J. Immunol., 6:511 (1976), which is hereby incorporated by reference.) This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth, and

to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described.

Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the protein or polypeptide of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 μl per site at six different sites. Each injected material will contain synthetic surfactant adjuvant pluronic polyols, or pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. Ultimately, the rabbits are euthenized with pentobarbital 150 mg/Kg IV. This and other procedures for raising polyclonal antibodies are disclosed in Harlow et. al., editors, Antibodies: A Laboratory Manual (1988), which is hereby incorporated by reference.

In addition to utilizing whole antibodies, binding portions of such antibodies can be used. Such binding portions include Fab fragments, $F(ab')_2$ fragments, and Fv fragments. These antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in Goding, Monoclonal Antibodies: Principles and Practice, New York:Academic Press, pp. 98-118 (1983), which is hereby incorporated by reference.

The present invention also relates to probes found either in nature or prepared synthetically by recombinant DNA procedures or other biological procedures. Suitable probes are molecules which bind to grapevine leafroll viral antigens identified by the monoclonal antibodies of the present invention. Such probes can be, for example, proteins, peptides, lectins, or nucleic acid probes.

The antibodies or binding portions thereof or probes can be administered to grapevine leafroll virus infected scion cultivars or rootstock cultivars. Alternatively, at least the binding portions of these antibodies can be sequenced, and the encoding DNA synthesized. The encoding DNA molecule can be used to transform plants together with a promoter which causes expression of the encoded antibody when the plant is infected by grapevine leafroll virus. In either case, the antibody or binding portion thereof or probe will bind to the virus and help prevent the usual leafroll response.

Antibodies raised against the proteins or polypeptides of the present invention or binding portions of these antibodies can be utilized in a method for detection of grapevine leafroll virus in a sample of tissue, such as tissue from a grape scion or rootstock. Antibodies or binding portions thereof suitable for use in the detection method include those raised against a helicase, an RNA-dependent RNA polymerase, an hsp70-related, an hsp90-related, or a coat protein or polypeptide in accordance with the present invention Any reaction of the sample with the antibody is detected using an assay system which indicates the presence of grapevine leafroll virus in the sample. A variety of assay systems can be employed, such as enzyme-linked immunosorbent assays,

radioimmunoassays, gel diffusion precipitin reaction assays, immunodiffusion assays, agglutination assays, fluorescent immunoassays, protein A immunoassays, or immunoelectrophoresis assays.

Alternatively, grapevine leafroll virus can be detected in such a sample using a nucleotide sequence of the DNA molecule, or a fragment thereof, encoding for a protein or polypeptide of the present invention. The nucleotide sequence is provided as a probe in a nucleic acid hybridization assay or a gene amplification detection procedure (e.g., using a polymerase chain reaction procedure). Any reaction with the probe is detected so that the presence of grapevine leafroll virus in the sample is indicated.

The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

EXAMPLES

Example 1 - Materials and Methods

Virus purification and dsRNA isolation. The NY1 isolate, which is also referred to as isolate GLRaV 109 by Golino, "The Davis Grapevine Virus Collection," Amer. J. Enol. Vitic, 43:200-205 (1992), a member of GLRaV-3 (Hu et al., "Characterization of Closterovirus-like Particles Associated with Grapevine Leafroll Disease," J. Phytopathol. (Berl.), 128:1-14 (1990) ("Hu (1990)") and Zee et al., "Cytopathology of Leafroll-Diseased Grapevines and the Purification and Serology of Associated Closteroviruslike Particles," Phytopathology, 77:1427-1434 (1987) ("Zee (1987)"), which are hereby incorporated by reference) was used throughout this work. Leafroll-diseased canes and mature leaves were collected from a vineyard in central

New York State, and kept at -20°C until used. GLRaV-3 virus particles were purified according to the method described by Zee (1987), which is hereby incorporated by reference, and modified later by Hu (1990), which is incorporated by reference. After two cycles of Cs_2SO_4 gradient purification, virus particles were observable from virus-enriched fractions by negative staining on an electron microscope.

The dsRNA was extracted from scraped bark/phloem tissue of canes as described in Hu (1990), which is hereby incorporated by reference. Briefly, total nucleic acid was extracted with phenol/chloroform; dsRNA was absorbed on a CF-11 cellulose column under 17% ethanol and eluted without ethanol. After two cycles of ethanol precipitation, dsRNA was analyzed by electrophoresis on a 6% polyacrylamide or 1% agarose gel. A high *Mr* dsRNA (~16 kb) along with several smaller *Mr* dsRNAs was consistently identified in leafroll diseased but not in healthy samples (Hu (1990), which is hereby incorporated by reference). The 16 kb dsRNA, which was presumably a replicative form of the virus, was purified further following separation on a low melting temperature-agarose gel (Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press (1989) ("Sambrook (1989)"), which is hereby incorporated by reference). The double-stranded nature of the dsRNA was confirmed after it was demonstrated to be resistant to DNase and RNase in high salt but sensitive to RNase in water (Hu (1990), which is hereby incorporated by reference).

cDNA synthesis and molecular cloning. Complementary DNA (cDNA) was prepared by the procedure of Gubler et al., "A Simple and Very Efficient Method for Generating cDNA Libraries," Gene, 25:263 (1983),

which is hereby incorporated by reference, and modified for dsRNA by Jelkmann et al., "Cloning of Four Plant Viruses from Small Quantities of Double-Stranded RNA," Phytopathology, 79:1250-1253 (1989), which is hereby incorporated by reference. Briefly, following denaturation of about 2 μg of dsRNA in 20 mM methylmercuric hydroxide (MeHg) for 10 min, the first-strand cDNA was synthesized by avian myeloblastosis virus ("AMV")-reverse transcriptase using random primers (Boehringer Mannheim, Indianapolis, IN). The second-strand cDNA was synthesized with DNA polymerase I while RNA templates were treated with RNase H. The cDNA was size-fractionated on a CL-4B Sepharose column and peak fractions, which contained larger molecular weight cDNA, were pooled and used for cloning. Complimentary DNA ends were blunted with T4 DNA polymerase, and *Eco* RI adapters were ligated onto a portion of the blunt-ended cDNA. After treatment with T4 polynucleotide kinase and removal of unligated adapters by spin column chromatography, the cDNA was ligated with lambda ZAPII/*Eco*R I prepared arms (Stratagene, La Jolla, CA). These recombinant DNAs were packaged *in vitro* with GIGAPACK II GOLD™ packaging extract according to the manufacturer's instruction (Stratagene). The packaged phage particles were used to infect bacteria, XL1-blue cells.

Screening the cDNA library. To select GLRaV-3 dsRNA specific cDNA clones, probes were prepared from UNI-AMP™ (Clontech, Palo Alto, CA) PCR-amplified cDNA. PCR-amplified GLRaV-3 cDNA was labeled with ^{32}P [a-dATP] by Klenow fragment of *E. coli* DNA polymerase I with random primers and used as a probe for screening the library (Feinberg et al., "A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity,"

Analytic Biochem., 132:6-13 (1983) ("Feinberg (1983)"), which is hereby incorporated by reference). Library screening was carried out by transferring plaques grown overnight onto GENESCREEN PLUS™ filters, following the manufacturer's instructions for denaturation, prehybridization, and hybridization (Dupont, Boston, MA). After washing, an autoradiograph was developed after exposing Kodak X-OMAT film to the washed filters overnight at -80°C. Bacteriophage recombinants were converted into plasmids (*in vivo* excision) following the manufacturer's instruction (Stratagene).

Identification of the coat protein gene was done by immunoscreening the cDNA library with GLRaV-3 specific polyclonal (Zee (1987), which is hereby incorporated by reference) and monoclonal (Hu (1990), which is hereby incorporated by reference) antibodies. Degenerate primer (5'GGNGGNGGNACNTTYGAYGTNTCN (SEQ. ID. No. 25), I=inosine, Y=T or C) generated from a conserved amino acid sequence in Motif C of the BYV HSP70 gene (p65) was used to select HSP70 positive clones. Further sequence extension was made possible by the clone walking strategy, which used sequences that flanked the sequence contig to probe the library for a clone that might contain an insert extending farther in either 5' or 3' direction.

Northern blot hybridization. Inserts from selected clones were labeled with ^{32}P[a-dATP] by Klenow fragment of *E. coli* DNA polymerase I (Feinberg (1983), which is hereby incorporated by reference) and used as probes to test their specific reactions to dsRNAs isolated from leafroll infected tissues. Double-stranded RNA isolated from GLRaV-3 infected vines was separated by electrophoresis on a 1% agarose gel (nondenatured condition), denatured with 50 mM NaOH, 0.6 M NaCl for 30 min at room temperature, and

neutralized with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5 for another 30 min. Denatured dsRNA was sandwich-blotted onto a GENESCREEN PLUS™ membrane. Prehybridization and hybridization were carried out in a manner similar to that described above. The membrane was washed and exposed to Kodak X-OMAT film, and an autoradiograph was developed.

Identification of immunopositive clones. For immunoscreening, plates with plaques appearing after 8-12 h incubation at 37°C were overlaid with a 10 mM isopropyl-β-D-thio-galactopyranoside ("IPTG") impregnated Nylon filters (GENESCREEN PLUS™) and incubated for an additional 3-4 h. After blocking with 3% bovine serum albumin ("BSA"), the blotted filter was incubated in a 1:1000 dilution of alkaline phosphatase-conjugated GLRaV-3 polyclonal antibody for 3 h at 37°C. Positive signals (purple dots) were developed by incubation of washed filters in a freshly prepared nitroblue tetrazolium ("NBT") and 5-bromo-4-chloro-3-indolyl phosphate ("BCIP") solution. To further confirm whether or not a true GLRaV-3 coat protein expression plaque was selected, a secondary immunoscreening was carried out by reinfection of bacterial XL1 Blue cells with an earlier selected plaque.

Western blot analysis. After secondary immunoscreening, GLRaV-3 antibody positive plaques were converted into plasmid, the pBluescript, by *in vivo* excision. Single colonies were picked up and cultured in LB medium with 100 μg/ml of ampicillin until mid-log growth. Fusion protein expression was induced by addition of 10 mM IPTG with an additional 3 h of incubation at 37°C. Bacteria was pelleted and denatured by boiling in protein denaturation buffer (Sambrook (1989), which is hereby incorporated by

reference). An aliquot of 5 µl denatured sample was loaded and separated by electrophoresis on a 12% SDS-polyacrylamide gel along with a prestained protein molecular weight marker (Bio-Rad, Hercules, CA). The separated proteins were transferred onto an Immobulon membrane (Millipore) with an electroblotting apparatus (Bio-Rad). After blocking with 3% BSA, the transferred membrane was incubated with 1:1,000 dilution of either GLRaV-3 polyclonal or monoclonal antibody alkaline phosphatase conjugate. A positive signal was developed after incubation of the washed membrane in NBT and BCIP.

PCR analysis. To analyze a cloned insert, an aliquot of a bacterial culture was used directly in PCR amplification with common vector primers (SK and KS). PCR-amplified product was analyzed by electrophoresis on an agarose gel.

Nucleotide sequencing and computer sequence analysis. Plasmid DNA, purified by either a CsCl method (Sambrook (1989), which is hereby incorporated by reference) or a modified mini alkaline-lysis/PEG precipitation procedure (Applied Biosystems' Instruction), was sequenced either with Sequenase version 2 kit following the manufacturer's instruction (US Biochemical, Cleveland, Ohio) or with Taq DYEDEOXY™ terminator cycle sequencing kit (Applied Biosystems, Inc.). Automated sequencing was conducted on an ABI373 automated sequencer at the New York State Agricultural Experiment Station in Geneva, New York.

Nucleotide sequences were analyzed using a Genetics Computer Group (GCG) sequence analysis software package (Madison, Wisconsin). Sequence fragments were assembled using Newgelstart to initiate the GCG fragment assembly system and to support automated fragment assembly in GCG Version 7.2.

Computer-assisted analysis of phylogenetic relationship. Amino acid sequences were either obtained from database Swiss-Prot or translated from nucleotide sequences obtained from GenBank. A phylogenetic tree depicting a relationship in the evolution of the GLRaV-3 coat protein sequence with respect to those of other filamentous plant viruses was generated using the Clustal Method of the DNASTAR's MegAlign program (Madison, Wisconsin). With the Clustal method, a preliminary phylogeny is derived from the distances between pairs of input sequences and the application of the UPGMA algorithm (Sneath et al., Numerical Taxonomy - The Principles and Practice of Numerical Taxonomy, Freeman Press (1973), which is hereby incorporated by reference) which guides the alignment of ancestral sequences. The final phylogeny is produced by applying the neighborhood joining method of Saitou et al., "The Neighbor Joining Method: A New Method for Reconstructing Phylogenetic Trees," Mol. Biol. Evol., 4:406-425 (1987), which is hereby incorporated by reference, to the distance and alignment data.

Nucleotide sequence and primer selection. The sequence fragment (Figure 2) selected for PCR has now been identified to be from nucleotides 9,364 to 10,011 of the incomplete GLRaV-3 genome (Figure 18). This sequence region encodes a short peptide which shares sequence similarity to HSP90 homologues of other closteroviruses (Figure 3). Selected primers and their designations are shown in Figure 2.

Sample preparation. These include 1) dsRNA, 2) purified virus, 3) partially purified virus, 4) proteinase K treated crude extract, and 5) immuno-capture preparation.

Isolation of dsRNA from leafroll infected grapevine tissues followed the procedure developed by Hu (1990), which is hereby incorporated by reference.

Virus purification was effected by the following procedure. An aliquot of 500 µl GLRaV-3-enriched fractions after two cycles of Cs_2SO_4 gradient was diluted with two volumes of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated on ice for 5 min. The reaction was then adjusted to a final concentration of 200 mM NaAc, pH 5.0, 0.5% SDS, and 200 µg/ml proteinase K and incubated at 37°C for 3 h. Viral RNA was extracted with phenol and chloroform, ethanol-precipitated, and resuspended in 50 µl of diethyl pyrocarbonate ("DEPC")-treated H_2O. For each 100 µl PCR reaction mixture, 1 µl of purified viral RNA was used as template.

Partially purified virus was prepared according to the virus purification procedure described in Hu (1990), which is hereby incorporated by reference, but only to the high speed centrifugation (27,000 rpm, 2 h) step without further Cs_2SO_4 gradient centrifugation. The pellet was resuspended in TE buffer and subjected to proteinase K treatment as described above. Viral RNA was extracted with phenol/chloroform and precipitated by ethanol. From 10 g of starting material, the pellet was resuspended in 200 µl of DEPC treated H_2O. A 1 µl aliquot of extracted RNA or its 10-fold dilution series (up to 10^{-5}) was used for reverse transcription-PCR ("RT-PCR").

Crude extract was treated with Proteinase K using the following procedure. Liquid nitrogen powdered grapevine bark/phloem tissue (100 mg) was macerated in 1 ml of virus extraction buffer (0.5 M Tris-HCl, pH 9.0, 0.01 M $MgSO_4$, 4% water insoluble

polyvinyl pyrrolidone ("PVP40"), 0.5% bentonite, 0.2% 2-mercaptoethanol, and 5% Triton X-100) (Zee (1987), which is hereby incorporated by reference). After a brief centrifugation (5,000 rpm, 2 min), 500 µl of supernatant was transferred into a new tube, adjusted to 100 µg/ml proteinase K, and incubated for 1 h at 55°C (Kawasaki, "Sample Preparation from Blood, Cells, and Other Fluids," in Innis et al., eds, PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990), which is hereby incorporated by reference). Following incubation, the preparation was boiled for 10 min to inactivate proteinase K and to denature the viral RNA. The upper clear phase was transferred into a new tube after a brief centrifugation. The viral RNA was precipitated with ethanol and resuspended in 100 µl of DEPC-treated H_2O. An aliquot of 1 µl proteinase K-treated crude extract or its 10-fold dilution series (up to 10^{-6}) was used.

The immuno-capture procedure was adapted from the method described by Wetzel et al., "A Highly Sensitive Immunocapture Polymerase Chain Reaction Method for Plum Pox Potyvirus Detection," J. Virol. Meth. 39:27-37 (1992) ("Wetzel (1992)"), which is hereby incorporated by reference. A 0.5 ml thin wall PCR tube was coated directly with 100 µl of 10 µg/ml purified gamma-globulin from GLRaV-3 antiserum (Zee (1987), which is hereby incorporated by reference) in ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, and 0.02% NaN_3) and incubated for 4 h at 30°C. After washing 3 times with PBS-Tween-20, the antibody coated tube was loaded with 100 µl of crude extract (1:10 or its 10-fold dilution series, up to 10^{-8}) prepared in ELISA extraction buffer (50 mM sodium citrate, pH 8.3, 20 mM sodium diethyldithiocarbonate ("DIECA"), 2% PVP 40K) and incubated at 30°C for 4 h.

After washing, a 25 μl aliquot of transfer buffer (10 mM Tris, pH 8.0, 1% Triton X-100) was added to the tube and vortexed thoroughly to release viral RNA.

RT-PCR. Initially, reverse transcription ("RT") and polymerase chain reaction ("PCR") were performed in two separate reactions. An aliquot of 20 μl of reverse transcription reaction mixture was prepared to contain 2 μl of 10X PCR buffer (Promega) (10 mM Tris-HCl, pH 8.3, 500 mM KCl, and 0.01% gelatin), 50 mM $MgCl_2$, 2 μl of 10 mM dNTP, 150 ng of 5' and 3' primers, 16 units of RNasin, 25 units of avian myeloblastosis virus ("AMV") reverse transcriptase, and 1 μl of a denatured sample preparation. The reverse transcription reaction was carried out at 37°C for 30 min. After denaturation by heating at 95°C for 5 min, an aliquot of PCR reaction mixture was added. This PCR reaction mixture (80 μl) contained 8 μl of 10X PCR buffer (Promega), 150 mM $MgCl_2$, 250 ng of each 5' and 3' primer, 1 μl of 10 mM dNTP, and 2.5 units of Taq DNA polymerase. The thermal cycling program was set as follows: a precycle at 92°C for 3 min; followed by 35 cycles of denaturation at 92°C, 1 min; annealing at 50°C, 1 min; and extension at 72°C, 2.5 min. The final extension cycle was set at 72°C for 5 min.

Because reverse transcriptase can work under the PCR buffer system, combination of RT and PCR would make RT-PCR in a single reaction (Ali et al., "Direct Detection of Hepatitis C Virus RNA in Serum by Reverse Transcription PCR," Biotechniques, 15:40-42 (1993) and Goblet et al., "One-Step Amplification of Transcripts in Total RNA Using the Polymerase Chain Reaction," Nucleic Acids Research, 17:2144 (1989), which are hereby incorporated by reference). The RT-PCR reaction mixture of 100 μl contains 10 μl of 10X PCR amplification buffer (Promega), 200 mM $MgCl_2$, 250 ng

each of primers, 3 μl of 10 mM dNTPs, 40 units of RNasin, 25 units of AMV or moloney-murine leukemia virus ("M-MLV") reverse transcriptase, 2.5 units of Taq DNA polymerase, and 1 μl of denatured sample preparation. The thermal cycling program was set as follows: one cycle of cDNA synthesis step at 37°C for 30 min, immediately followed by the PCR cycling parameters described above.

Nested PCR. Inconsistent results obtained from a single round of PCR amplification prompted an investigation into the feasibility of Nested PCR. Initial PCR amplification was performed with an external primer set (93-110 & 92-98) (Figure 2). A PCR product of 648 bp was consistently observed from dsRNA as template, but the expected PCR product was not consistently observed in samples prepared from proteinase K-treated crude extract or immuno-capture sample preparation. Consequently, additional PCR amplification with an internal primer set (93-25 & 93-40) was carried out by adding 5 μl of the first external primer-amplified PCR product into a freshly prepared 100 μl PCR reaction mixture. The PCR cycling parameters were the same as described above.

Example 2 - Virus Purification and dsRNA Isolation.

GLRaV-3 virus particles were purified directly from field collected samples of infected grapevines. Attempts to use genomic RNA for cDNA cloning failed due to low yield of virus particles with only partial purity (Figure 1). However, under an electron microscope, virus particles were shown to be decorated by GLRaV-3 antibody. The estimated coat protein molecular weight of 41K agreed with an earlier study (Hu (1990), which is hereby incorporated by reference). Because of low yield in virus

purification, dsRNA isolation was further pursued. Based on the assumption that high *Mr* dsRNA (16 kb) is the replicative form of the GLRaV-3 genomic RNA, this high *Mr* dsRNA was separated from other smaller ones by electrophoresis (Figure 5), purified from a low melting temperature agarose gel, and used for cDNA synthesis.

Example 3 - cDNA Synthesis, Molecular Cloning, and Analysis of cDNA Clones.

First-strand cDNA was synthesized with AMV reverse transcriptase from purified 16 kb dsRNA which had been denatured with 10 mM MeHg. Only random primers were used to prime the denatured dsRNA because several other closteroviruses (BYV, CTV, and LIYV) have been shown to have no polyadenylated tail on the 3' end (Agranovsky et al., "Nucleotide Sequence of the 3'-Terminal Half of Beet Yellows Closterovirus RNA Genome Unique Arrangement of Eight Virus Genes," Journal of General Virology, 72:15-24 (1991) ("Agranovsky (1991)"), Agranovsky et al., "Beet Yellows Closterovirus: Complete Genome Structure and Identification of a Papain-like Thiol Protease," Virology, 198:311-324 (1994) ("Agranovsky (1994)"), Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology, 208:511-520 (1995) ("Karasev (1995)"), Klaassen et al., "Genome Structure and Phylogenetic Analysis of Lettuce Infectious Yellows Virus, A Whitefly-Transmitted, Bipartite Closterovirus," Virology, 208:99-110 (1995) ("Klaassen (1995)"), and Pappu et al., "Nucleotide Sequence and Organization of Eight 3' Open Reading Frames of the Citrus Tristeza Closterovirus Genome," Virology, 199:35-46 (1994) ("Pappu (1994)"), which are hereby incorporated by reference). After second-strand cDNA synthesis, the cDNA was size-fractionated on a CL-

4B Sepharose column and peak fractions which contained larger molecular weight cDNA were pooled and used for cloning. An autoradiograph of this pooled cDNA revealed cDNA of up to 4 kb in size. A bacteriophage cDNA library was prepared after cloning of the synthesized cDNA into the cloning vector, lambda ZAPII.

A lambda ZAPII library was prepared from cDNA that was synthesized with random primed, reverse transcription of GLRaV-3 specific dsRNA. Initially, white/blue color selection in IPTG/X-gal containing plates was used to estimate the ratio of recombination. There were 15.7% white plaques or an estimate of 7 X 10^4 GLRaV-3 specific recombinants in this cDNA library. The library was screened with probes prepared from UNI-AMP™ PCR-amplified GLRaV-3 cDNA. More than 300 clones with inserts of up to 3 kb were selected after screening the cDNA library with probe prepared from UNI-AMP™ PCR-amplified GLRaV-3 cDNA. In Northern blot hybridization, a probe prepared from a clone insert, pC4, reacted strongly to the 16 kb dsRNA as well as to several other smaller *Mr* dsRNAs. Such a reaction was not observed with nucleic acids from healthy grape nor to dsRNA of CTV (Figure 4).

Example 4 - Selection and Characterization of Immunopositive Clones

A total of 6 X 10^4 plaques were immunoscreened with GLRaV-3 specific polyclonal antibody. Three cDNA clones, designated pCP5, pCP8-4, and pCP10-1, produced proteins that reacted to the polyclonal antibody to GLRaV-3 (Figure 6). GLRaV-3 antibody specificity of the clones was further confirmed by their reaction to GLRaV-3 monoclonal antibody. PCR analysis of cloned inserts showed that a similar size of PCR product (1.0-1.1 kb) was cloned in

each immunopositive clone (Figure 7). However, various sizes of antibody-reacting protein were produced from each clone, which suggested that individual clones were independent and contained different segments of the coat protein gene (Figure 8). The *Mr* of immunopositive fusion protein from clone pCP10-1 was estimated to be 50K in SDS-PAGE, which was greater than the native coat protein of 41K (compare lanes 1 to 4 in Figure 8). Immunopositive proteins produced in clone pCP5 (Figure 8, lane 2) and pCP8 (Figure 8, lane 3) were different in size and smaller than the native coat protein. Clone pCP5 produced a GLRaV-3 antibody-reacting protein of 29K. Clone pCP8-4, however, produced an antibody-reacted protein of 27K. Similar banding patterns were observed when either polyclonal (Figure 8 A) or monoclonal (Figure 8 B) antibodies were used in Western blots. These results further substantiated the proposition that these cDNA clones contained coding sequences of the GLRaV-3 coat protein gene.

Example 5 - Nucleotide Sequencing and Identification of the Coat Protein Gene

Both strands of the three immunopositive clones were sequenced at least twice. A multiple sequence alignment of these three clones overlapped and contained an incomplete ORF lacking the 3' terminal sequence region. The complete sequence of this ORF was obtained by sequencing an additional clone, pA6-8, which was selected by using the clone walking strategy. The complete ORF potentially encoded a protein of 313 amino acids with a calculated *Mr* of 34,866 (p35) (Figures 9 and 10). Because this ORF was derived from three independent clones after screening with GLRaV-3 coat protein specific antibody, it was identified as

the coat protein gene of GLRaV-3. A multiple amino acid sequence alignment of p35 with the coat proteins of other closteroviruses, including BYV, CTV, and LIYV, is presented in Figure 11. The typical consensus amino acid residues (S, R, and D) of the coat proteins of the filamentous plant viruses (Dolja et al., "Phylogeny of Capsid Proteins of Rod-Shaped and Filamentous RNA Plant Viruses Two Families with Distinct Patterns of Sequence and Probably Structure Conservation," Virology, 184:79-86 (1991) ("Dolja (1991)"), which is hereby incorporated by reference), which may be involved in salt bridge formation and the proper folding of the most conserved core region (Boyko et al., "Coat Protein Gene Duplication in a Filamentous RNA Virus of Plants," Proc. Natl. Acad. Sci. U.S.A., 89:9156-9160 (1992) ("Boyko (1992)"), which is hereby incorporated by reference), were also preserved in the p35. Phylogenetic analysis of the GLRaV-3 coat protein amino acid sequence with respect to the other filamentous plant viruses placed GLRaV-3 into a separate but closely related branch of the closterovirus (Figure 12). Direct sequence comparison of GLRaV-3 coat protein with respect to other closterovirus coat proteins or their diverged copies by the GCG Pileup program demonstrated that at the nucleotide level, GLRaV-3 had its highest homology to BYV (41.5%) and CTV (40.3%). At the amino acid level, however, the highest percentage similarity were to the diverged copies of coat protein, with 23.5% identity (46.5% similarity) to CTV p26 and 22.6% (44.3% similarity) to BYV p24.

Example 6 - Identification of a Possible Coat Protein Translation Initiation Site

Various sizes of GLRaV-3 specific antibody-reacted proteins were produced by three immunopositive

clones in *E. coli* (Figure 8). Sequences of these clones overlapped and encoded a common ORF that was identified as the coat protein gene (Figure 9). In searching for possible translation regulatory elements, sequence analysis beyond the coat protein coding region revealed a purine rich sequence, -uGAGuGAAcgcgAUG- (SEQ. ID. No. 26), which was similar to the Shine-Dalgarno sequence (uppercase letters) (Shine et al., "The 3'-Terminal Sequence of *Escherichia Coli* 16S Ribosomal RNA: Complementarity to Nonsense Triplets and Ribosome Binding Sites," Proc. Nat. Acad. Sci. U.S.A., 71:1342-1346 (1974), which is hereby incorporated by reference), upstream from the coat protein initiation site (AUG). This purine rich sequence may serve as an alternative ribosome entry site for the translation of the GLRaV-3 coat protein gene in *E. coli*. If this first AUG in the ORF was to serve for the actual coat protein translation, the ribosomal entry site must be located in this purine rich region because an in-frame translation stop codon (UGA) was only nine nucleotides upstream from the coat protein gene translation initiation site (AUG). Analysis of nucleotide sequence beyond the cloned insert into the vector sequence of clone pCP8-4 and pCP10-1 provided direct evidence that the fusion protein was made from the N-terminal portion of coat protein and C-terminal portion of β-galactosidase (16.5K). Further analysis of sequence around the selected AUG initiation codon of the coat protein gene revealed a consensus sequence (-GnnAUGG-) that favored the expression of eucaryotic mRNAs (Kozak, "Comparison of Initiation of Protein Synthesis in Procaryotes, Eucaryotes, and Organelles," Microbiological Reviews, 47:1-45 (1983) and Kozak, "Point Mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by

Eukaryotic Ribosomes," Cell, 44:283-292 (1986), which are hereby incorporated by reference).

Nucleotide sequence analysis of three immunopositive clones revealed overlapping sequences and an ORF that covers about 96% of the estimated coat protein gene (Figure 9). The complete ORF was obtained after sequencing of an additional clone (pA6-8) that was selected by the clone walking strategy. Identification of this ORF as the coat protein gene was based upon its immunoreactivity to GLRaV-3 polyclonal and monoclonal antibodies, the presence of filamentous virus coat protein consensus amino acid residues (S, R, and D), and the identification of a potential translation initiation site. The calculated coat protein molecular weight (35K) is smaller than what was estimated on SDS-PAGE (41K). This discrepancy in molecular weight between computer-calculated and SDS-PAGE estimated falls in the expected range. However, direct evidence by micro-sequencing of the N-terminal coat protein sequence was not possible due to the difficulties in obtaining sufficient amounts of purified virus.

The estimated coat protein Mr of GLRaV-3 and another grape closterovirus-like designated GLRaV-1 are larger than the 22-28K coat protein range reported for other well characterized closteroviruses, such as BYV, CTV, and LIYV (Agranovsky (1991); Bar-Joseph et al., "Closteroviruses," CMI/AAB, No. 260 (1982), Klaassen et al., "Partial Characterization of the Lettuce Infectious Yellows Virus Genomic RNAs, Identification of the Coat Protein Gene and Comparison of its Amino Acid Sequence with Those of Other Filamentous RNA Plant Viruses," Journal of General Virology, 75:1525-1533 (1994); (Martelli et al., "Closterovirus, Classification and Nomenclature of Viruses, Fifth

Report of the International Committee on Taxonomy of Viruses," in Archieves of Virology Supplementum 2, Martelli et al., eds., New York: Springer-Verlag Wein, pp. 345-347 (1991) ("Martelli (1991)"); and Sekiya et al., "Molecular Cloning and Nucleotide Sequencing of the Coat Protein Gene of Citrus Tristeza Virus," Journal of General Virology, 72:1013-1020 (1991), which are hereby incorporated by reference). Hu (1990), which is hereby incorporated by reference, suggested a possible coat protein dimer. Our sequence data, however, do not support this suggestion. First, the size of the coat protein is only 35K, which is smaller than what would be expected as a coat protein dimer. Second, a multiple sequence alignment of N-terminal half and C-terminal half of GLRaV-3 coat protein with the coat proteins of other closteroviruses showed that the filamentous virus coat protein consensus amino acid residues (S, R, and D) are only present in the C-terminal portion, but not in the N-terminal portion of the coat protein.

Example 7 - Primer Selection.

Primers were selected based on the nucleotide sequence of clone pC4 that had been shown to hybridize to GLRaV-3 dsRNAs on a Northern hybridization (Figure 4). The 648 bp sequence amplified by PCR was identified as nucleotides 9,364 to 10,011 of the incomplete GLRaV-3 genome (Figure 18). This sequence fragment encodes a short peptide which shows some degree of amino acid sequence similarity to heat shock protein 90 (HSP90) homologues of other closteroviruses, BYV, CTV, and LIYV (Figure 3). Two sets of primer sequences and their designations (external, 93-110 & 92-98, and internal, 93-25 & 93-40) are shown in Figure 2. Effectiveness of synthesized primers to amplify the

expected PCR product was first evaluated on its respective cDNA clone, pC4 (Figure 13, lane 11).

Example 8 - Development of a Simple and Effective PCR Sample Preparation.

Initially, purified dsRNA was used in a RT-PCR reaction. Expected size of PCR product of 219 bp was consistently observed with the internal set of primers (Figure 13, lane 10). To test whether or not these primers derived from GLRaV-3 specific dsRNA sequence is in fact the GLRaV-3 genome sequence, RNA extracted from a highly purified virus preparation was included in an assay. As expected, PCR products with similar size (219 bp) were observed in cloned plasmid DNA (pC4) (Figure 13, lane 11), dsRNA (Figure 13, lane 10) as well as purified viral RNA (Figure 13, lane 9). This PCR result was encouraging as it was the first evidence to suggest that dsRNA isolated from leafroll-infected tissue may actually be derived from the GLRaV-3 genome. However, PCR sample preparations from the purified virus procedure are too complicated to be used for leafroll diagnosis. Further simplification of sample preparations was made possible by using viral RNA extracted from a partially purified virus preparation. This partially purified virus preparation was again shown to be effective in RT-PCR (Figure 13). Sensitivity of RT-PCR was further evaluated with 10-fold serial dilution (up to 10^{-5}) of a sample. The expected PCR product of 219 bp in a partially purified virus preparation was observable up to the 10^{-3} dilution (Figure 13, lane 4). Although RT-PCR was shown again to work with partially purified virus preparations, this method of sample preparation was still too complicated to be used in a routine disease diagnosis. However, over 10 attempts to directly use

crude extract for RT-PCR were unsuccessful. Proteinase K-treated crude extract was by far the most simple and still effective for RT-PCR. Therefore, the proteinase K-treated crude extract was used to evaluate RT-PCR for its ability to detect GLRaV-3.

Example 9 - RT-PCR

With proteinase K-treated crude extract prepared from scraped phloem tissue collected from a typical leafroll infected vine (Doolittle's vineyard, New York), a PCR product of 219 bp was readily observable. However, application of this sample preparation method to test other field collected samples (USDA, PGRU, Geneva, NY) was disappointing. With different batches of sample preparations, a range of 3 to 10 out of 12 ELISA positive samples were shown to have the expected PCR products. To determine whether or not these inconsistent results were due to some kinds of enzyme (reverse transcriptase or Taq DNA polymerase) inhibition presented in the proteinase K-treated crude extract, increasing amounts of a sample were added into an aliquot of 100 μl PCR reaction mixture. Figure 14 shows that PCR products of 219 bp were readily observed from samples of 0.1 μl (lane 1) and 1 μl (lane 2) but not from 10 μl (lane 3). Presumably, sufficient amount of enzyme inhibitors was present in the 10 μl of this sample.

Example 10 - Immuno-capture RT-PCR

The immuno-capture method further simplified sample preparation by directly using crude extracts that were prepared in the standard ELISA extraction buffer. Immuno-capture RT-PCR ("IC RT-PCR") tests were initially performed with the internal primer set, and the expected PCR product of 219 bp was observable from

a typical leafroll infected sample. However, using this PCR method to test a range of field collected ELISA positive samples, inconsistent results were again experienced. In a PCR test performed with the external primer set, only five out of seven field collected ELISA positive samples were shown to amplify the expected PCR product (648 bp) (Figure 15 A). Meanwhile, the expected PCR product was consistently observed in dsRNA (Figure 15 A, lane 10), but such product was never observed in the healthy control (Figure 15 A, lane 9). In this case, however, the expected PCR product was not observable in a sample prepared by proteinase K-treated crude extract (Figure 15 A, lane 8).

Example 11 - Nested PCR

As described above, inconsistency of RT-PCR was experienced with samples prepared either by the proteinase K-treated or by the immuno-capture methods. If this PCR technique is to be used in the practical disease diagnosis, a consistent and repetitive result is desirable. Thus, the Nested PCR method was introduced. Although an expected PCR product of 648 bp from the first PCR amplification with the external primer set was not always observable (Figure 15 A), in a Nested PCR amplification with the internal primer set, the expected 219 bp PCR product was consistently observed from all seven ELISA positive samples (Figure 15 B). These similar products were also observed either in dsRNA (Figure 15 B, lane 10) or in the proteinase K-treated crude extract (Figure 15 B, lane 8) but, again, not in a healthy control (Figure 15 B, lane 9). To determine the sensitivity of Nested PCR with samples prepared either by proteinase K-treated or by immuno-capture methods, Nested PCR and ELISA were

performed simultaneously with samples prepared from a 10-fold dilution series. The sensitivity of Nested PCR was shown to be 10^{-5} in proteinase K-treated crude extract (Figure 16 A), and was more than 10^{-8} (the highest dilution point in this test) in an immuno-capture preparation (Figure 16 B). With similar sample preparations, sensitivity for ELISA was only 10^{-2}.

Example 12 - Validation of PCR with ELISA and indexing

To determine whether or not the PCR-based GLRaV-3 detection method described in this study has a potential practical implication for grapevine leafroll disease diagnosis, a validation experiment with plants characterized thoroughly by ELISA and indexing is necessary. Several grapevines collected at USDA-PGRU at Geneva, New York that have been well characterized by 3-year biological indexing and by ELISA were selected for validation tests. A perfect correlation was observed between ELISA positive and PCR positive samples, although there was some discrepancy over indexing which suggested that other types of closteroviruses may also be involved in the grapevine leafroll disease (Table 2).

TABLE 2

Sample #	Accession #	ELISA *	RT-PCR	Indexing
1	476.01	1.424 (+)	+	+
2	447.01	0.970 (+)	+	+
3	123.01	1.101 (+)	+	+
4	387.01	>1.965(+)	+	+
5	80.01	>2.020(+)	+	+
6	244.01	>2.000(+)	+	+
7	441.01	>2.000(+)	+	+
8	510.01	0.857 (+)	+	+
9	536.01	0.561 (+)	+	+
10	572.01	>2.000(+)	+	+
11	468.01	>2.000(+)	+	+
12	382.01	>2.000(+)	+	+
13	NY1	0.656 (+)	+	+
14	Healthy	0.002 (-)	-	-

Plus (+) and Minus (-) represent positive and negative reactions, respectively. For ELISA an OD_{405nm} that was at least twice higher than a healthy control, and more than 0.100 was regarded as positive.

PCR technology has been applied to detect viruses, viroids and phytoplasmas in the field of plant pathology (Levy et al., "Simple and Rapid Preparation of Infected Plant Tissue Extracts for PCR Amplification of Virus, Viroid and MLO Nucleic Acids," Journal of Virological Methods, 49:295-304 (1994), which is hereby incorporated by reference). However because of the presence of enzyme inhibitors (reverse transcriptase and/or Taq DNA polymerase) in many plant tissues, a lengthy and complicated procedure is usually required to prepare a sample for PCR. In studies of PCR detection of grapevine fanleaf virus, Rowhani et al., "Development of a Polymerase Chain Reaction Technique for the Detection of Grapevine Fanleaf Virus in Grapevine Tissue," Phytopathology, 83:749-753 (1993), which is hereby incorporated by reference, have already observed an enzyme inhibitory phenomenon. Substances such as phenolic compounds and polysaccharides in grapevine tissues were suggested to be involved in

enzyme inhibition. Present work further confirmed this observation. One of the objectives in the present study was to develop a sound practical procedure of sample preparation to eliminate this inhibitory problem for PCR detection of GLRaV-3 in grapevine tissues. Although the expected PCR product was consistently observed from samples of dsRNA, purified virus and partial purified virus, proteinase K-treated crude extract and immuno-capture methods were the simplest and were still effective. Samples prepared with proteinase K-treated crude extract have an advantage over others in that hazardous organic solvents, such as phenol and chloroform, are avoided. However, care must be taken in the sample concentration because the reaction can be inhibited by adding too much grapevine tissue (see lane 3 in Figure 14). Minafra et al., "Sensitive Detection of Grapevine Virus A, B, or Leafroll-Associated III from Viruliferous Mealybugs and Infected Tissue by cDNA Amplification," Journal of Virological Methods, 47:175-188 (1994) ("Minafra (1994)"), which is hereby incorporated by reference, reported the successful PCR detection of grapevine virus A, grapevine virus B, and GLRaV-3 with crude saps prepared from infected grapevine tissues, this method of sample preparation was, however, not effective in the present study. The similar primers used by Minafra (1994), which is hereby incorporated by reference, were, however, able to amplify the expected size of PCR products from dsRNA of the NY1 isolate of GLRaV-3.

Immuno-capture is another simple and efficient method of sample preparation (Wetzel (1992), which is hereby incorporated by reference). First, crude ELISA extracts can be used directly for RT-PCR. Second, it provides not only a definitive answer, but may also be an indication to a virus serotype. Third,

with an immuno-capture step, virus particles are trapped by an antibody, and inhibitory substances may be washed away. Nested PCR with samples prepared by the immuno-capture method is 10^3 times more sensitive than with samples prepared by proteinase K-treated crude extract. However, this approach requires a virus specific antibody. For some newly discovered or hard to purify viruses, a virus specific antibody might not be always available. More specifically, there are at least six serologically distinctive closteroviruses associated with grapevine leafroll disease (Boscia (1995)), which is hereby incorporated by reference).

Example 13 - Nucleotide Sequence and Open Reading Frames

A lambda ZAPII library was prepared from cDNA that was synthesized with random primed, reverse transcription of GLRaV-3 specific dsRNA. Initially, white/blue color selection in IPTG/X-gal containing plates was used to estimate the ratio of recombination. There were 15.7% white plaques or an estimate of 7 X 10^4 GLRaV-3 specific recombinants in this cDNA library. The library was screened with probes prepared from UNI-AMP™ PCR-amplified GLRaV-3 cDNA. More than 300 clones with inserts of up to 3 kb were selected after screening the cDNA library with probe prepared from UNI-AMP™ PCR-amplified GLRaV-3 cDNA. In Northern blot hybridization, a probe prepared from a clone insert, pC4, reacted strongly to the 16 kb dsRNA as well as to several other smaller *Mr* dsRNAs. Such a reaction was not observed with nucleic acids from healthy grape nor to dsRNA of CTV (Figure 4).

Sequencing work began with clone pB3-1 that was selected after screening the library with HSP70 degenerated primer (5'G-G-I-G-G-I-G-G-I-A-C-I-T-T-Y-G-

A-Y-G-T-I-T-C-I (SEQ. ID. No. 25)). Other clones that were chosen for nucleotide sequencing were selected by the clone walking strategy. The nucleotide sequencing strategy employed was based on terminal sequencing of random selected clones assisted with GCG fragment assembly program to assemble and extend the sequence contig. The step-by-step primer extension method was used to sequence the internal region of a selected clone. A total of 54 clones were selected for sequencing. Among them, 16 clones were completely sequenced on both DNA strands (Figure 17).

A total of 15,227 nucleotides were sequenced so far (Figure 18), which potentially encompass nine open reading frames (ORFs) (Figure 19), designated as ORFs 1a, 1b, and 2 to 8. The sequenced region was estimated to cover about 80% of the complete GLRaV-3 genome. Major genetic components, such as helicase (ORF 1a), RdRp (ORF 1b), HSP70 homologue (ORF 4), HSP90 homologue (ORF 5) and coat protein (ORF 6) were identified.

ORF 1a was an incomplete ORF from which the 5' terminal portion has yet to be cloned and sequenced. The sequenced region presented in Figures 18 and 19 represents approximately two-thirds of the expected ORF 1a , as compared to the ORF 1a from BYV, CTV, and LIYV. The partial ORF 1a was terminated by the UGA stop codon at positions 4,165-4,167; the respective product consisted of 1,388 amino acid residues and had a deduced *Mr* of 148,603. Database searching indicated that the C-terminal portion of this protein shared significant similarity with the Superfamily 1 helicase of positive-strand RNA viruses. Comparison of the conserved domain region (291 amino acids) showed a 38.4% identity with an additional 19.7% similarity between GLRaV-3 and BYV and a 32.4% identity with an

additional 21.1% similarity between GLRaV-3 and LIYV (Table 3). Six helicase conserved motifs of Superfamily 1 helicase of positive-strand RNA viruses (Hodgman, "A New Superfamily of Replicative Proteins," Nature, 333:22-23 (Erratum 578) (1988) and Koonin et al., "Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences," Critical Reviews in Biochemistry and Molecular Biology, 28:375-430 (1993), which are hereby incorporated by reference) were also retained in GLRaV-3 (Figure 20). Analysis of the phylogenetic relationship in helicase domains between GLRaV-3 and the other positive-strand RNA viruses placed GLRaV-3 along with the other closteroviruses, including BYV, CTV, and LIYV, into the "tobamo" branch of the alphavirus-like supergroup (Figure 21).

Table 3

Virus	Helicase nt	Helicase aa	RdRp nt	RdRp aa	p5K nt	p5K aa	HSP70 nt	HSP70 aa	HSP90 nt	HSP90 aa	CP nt	CP aa
BYV	37.7	38.4 (58.1)	44.5	41.2 (61.0)	42.0	30.4 (47.8)	43.5	28.6 (48.0)	40.5	21.7 (51.0)	41.5	20.3 (43.7)
CTV	45.3	36.3 (55.2)	44.0	40.1 (62.2)	42.8	20.0 (48.9)	43.7	28.7 (49.3)	38.6	17.5 (43.5)	40.3	20.5 (41.9)
LIYV	44.9	32.4 (53.5)	46.2	35.9 (56.4)	45.8	17.9 (46.2)	43.9	28.2 (46.9)	39.3	16.7 (36.8)	36.3	17.8 (41.1)

Nucleotide ("nt") and amino acid ("aa") sequence similarity was calculated from perfect matches after aligning with the GCG program GAP; the percentages in parentheses are the percentages calculated by the GAP program, which employs a matching table based on evolutionary conservation of amino acids (Devereux et al., "A Comprehensive Set of Sequence Analysis Programs for the VAX," Nucleic Acids Res., 12:387-395 (1984), which is hereby incorporated by reference). The sources for the BYV, CTV, and LIYV sequences were, respectively, Agranovsky (1994), Karasev (1995), and Klaassen (1995), which are hereby incorporated by reference.

ORF 1b overlapped the last 113 nucleotides of ORF 1a and terminated at the UAG codon at positions 5780 to 5782. This ORF encoded a protein of 536 amino acid residues, counting from the first methionine codon and had a calculated *Mr* of 61,050 (Figures 18 and 19). Database screening of this protein revealed a significant similarity to the Supergroup 3 RdRp of the positive-strand RNA viruses. Sequence comparison of GLRaV-3 with BYV, LIYV, and CTV over a 313-amino acid sequence fragment revealed a striking amino acid sequence similarity among eight conserved motifs (Figure 22). The best alignment was with BYV, with 41.2% identity and 19.8% additional similarity while the least alignment was with LIYV, with 35.9% identity and 20.5% additional similarity (Table 3). Analysis of

phylogenetic relationships of the RdRp domains of the alphavirus-like supergroup viruses again placed GLRaV-3 into a "tobamo" branch along with other closteroviruses, BYV, CTV, BYSV, and LIYV (Figure 23).

Publications on BYV, CTV, and LIYV have proposed that ORF 1b is expressed via a +1 ribosomal frameshift (Agranovsky (1994), Dolja et al., "Molecular Biology and Evolution of Closteroviruses: Sophisticated Build-up of Large RNA Genomes," Annual Review of Phytopathology, 32:261-285 (1994) ("Dolja (1994)"), Karasev (1995), and Klaassen (1995), which are hereby incorporated by reference). Direct nucleotide sequence comparison was performed within the ORF1a/1b overlap of GLRaV-3 with respect to BYV, CTV, or LIYV. An apparently significant similarity was observed only to LIYV (Figure 24), and not to BYV or CTV. The so-called "slippery" GGGUUU sequence and the stem-and-loop structure that were proposed to be involved in the BYV frameshift was absent from the GLRaV-3 ORF1a/1b overlap. The frameshift within the GLRaV-3 ORF 1a/1b overlap was selected based on an inspection of the C-terminal portion of the helicase alignment and the N-terminal portion of the RdRp alignment between GLRaV-3 and LIYV (Figure 24). The GLRaV-3 ORF 1a/1b frameshift was predicted to occur in the homologous region of the LIYV genome, and was also preceded by a repeat sequence (GCTT) (Figure 24). Unlike LIYV, this repeat sequence was not a tandem repeat and was separated by one nucleotide (T) in GLRaV-3. The frameshift was predicted to occur at CACA (from His to Thr) in GLRaV-3 rather than slippery sequence AAAG in LIYV. However, additional experiments on *in vitro* expression of GLRaV-3 genomic RNA are needed in order to determine whether or not a large fusion protein is actually produced.

ORF 2 potentially encoded a small peptide of 51 amino acids with a calculated *Mr* of 5,927. Database searching did not reveal any obvious protein matches within the existing Genbank (Release 84.0).

Intergenic regions of 220 bp between ORF 1b and ORF 2 and 1,065 bp between ORF 2 and ORF 3 were identified. There is no counterpart in BYV or LIYV genomes; instead, an ORF of 33K in CTV (Karasev et al., "Screening of the Closterovirus Genome by Degenerate Primer-Mediated Polymerase Chain Reaction," Journal of General Virology, 75:1415-1422 (1994), which is hereby incorporated by reference) or 32K in LIYV (Klaassen (1995), which is hereby incorporated by reference) is observed over this similar region.

ORF 3 encoded a small peptide of 45 amino acids with a calculated *Mr* of 5,090 (p5K). Database searching revealed that it was most closely related to the small hydrophobic, transmembrane proteins of BYV (6.4K), CTV (6K), and LIYV (5K) (Fig 25). Individual comparison (Table 3) showed that LIYV was its most close relative (45.8%) at the nucleotide level and BYV was the most homologous (30.4%) at the amino acid level.

ORF 4 potentially encoded a protein of 549 amino acids with a calculated *Mr* of 59,113 (p59) (Figures 18 and 19). Database screening revealed a significant similarity to the HSP70 family, the p65 protein of BYV, the p65 protein of CTV, and the p62 protein of LIYV. A multiple amino acid sequence alignment of GLRaV-3 p59 with HSP70 analogs of other closteroviruses showed a striking sequence similarity among eight conserved motifs (A-H) (Figure 26). Functionally important motifs (A-C) that are characteristic of all proteins containing the ATPase domain of the HSP70 type (Bork et al., "An ATPase Domain Common to Prokaryotic Cell Cycle Proteins, Sugar

Kinases, Actin, and HSP70 Heat Shock Proteins," Proc. Natl. Acad. Sci. U.S.A., 89:7290-7294 (1992), which is hereby incorporated by reference) were also preserved in GLRaV-3 p59 (Figure 26), which suggested that this HSP70 chaperon-like protein may also possess ATPase activity on its N-terminal domain and protein-protein interaction on its C-terminal domain (Dolja (1994), which is hereby incorporated by reference). Analysis of the phylogenetic relationship of p59 of GLRaV-3 with HSP70-related proteins of other closteroviruses (BYV, CTV, and BYSV) and cellular HSP70s again placed the four closteroviruses together and the rest of the cellular HSP70s on the other branches (Figure 27). Although several closterovirus HSP70-related proteins are closely related to each other and distant from other cellular members of this family, inspection of the phylogenetic tree (Figure 27) suggested that GLRaV-3 may be an ancestral closterovirus relatively early in evolution as predicted by Dolja (1994), which is hereby incorporated by reference, because GLRaV-3 was placed in between closteroviruses and the other cellular HSP70 members.

ORF 5 encoded a protein of 483 amino acids with a calculated *Mr* of 54,852 (p55) (Figures 18 and 19). No significant sequence homology with other proteins was observed in the current database (GenBank, release 84.0). Direct comparison with other counterparts (p61 of CTV, p64 of BYV, and p59 of LIYV) of closteroviruses revealed some degree of amino acid sequence similarity, with 21.7% to BYV, 17.5% to CTV, and 16.7% to LIYV, respectively (Table 3, Figure 28). Two conserved regions of HSP90 previously described in BYV and CTV (Pappu (1994), which is hereby incorporated by reference) were identified in the p55 of GLRaV-3 (Figure 28).

The data in this ORF has been extensively described. ORF 6 encoded a protein of 313 amino acids with a calculated *Mr* of 34,866 (p35) (Figures 18 and 19). The fact that this ORF was encoded by three overlapping GLRaV-3 immunpositive clones suggests that it may contain the coat protein gene of GLRaV-3. Alignment of the product of ORF 6 (p35) with respect to BYV, CTV, and LIYV, is presented in Figure 11. The typical consensus amino acid residues (S, R, and D) of the coat protein of the filamentous plant viruses (Dolja (1991), which is hereby incorporated by reference), which may be involved in salt bridge formation and the proper folding of the most conserved core region (Boyko (1992), which is hereby incorporated by reference), were also retained in the p35 (Figure 11). Individual sequence comparison showed the highest similarity to CTV (20.5%) and BYV (20.3%), and the lowest similarity to LIYV (17.8%). Analysis of phylogenetic relationships with other filamentous plant viruses placed GLRaV-3 into a separate, but a closely related branch of closteroviruses (Figure 12).

ORF 7 encoded a protein of 477 amino acids with a calculated *Mr* of 53,104 (p53) (Figures 18 and 19). Based on the presence of conserved amino acid sequences, this protein is designated as grapevine leafroll coat protein repeat (p53).

ORF 8 encoded an unidentified polypeptide having a calculated *Mr* of 21,148 (p21).

ORF 9 encoded an unidentified polypeptide having a calculated *Mr* of 19,588 (p20).

ORF 10 encoded an unidentified polypeptide having a calculated *Mr* of 19,653 (p20).

ORF 11 encoded an unidentified polypeptide having a calculated *Mr* of 6,963 (p7).

In the present study, many GLRaV-3 dsRNA specific cDNA clones were identified using a probe generated from UNI-AMP™ PCR-amplified cDNA. Using UNI-AMP™ adapters and primers (Clontech) in PCR has several advantages. First, it is not necessary to know the nucleotide sequence of an amplified fragment. Second, cDNA can be amplified in sufficient amounts for specific probe preparation. In general, cDNA amplified by PCR using UNI-AMP™ primers and adapters could be used for cloning as well as a probe for screening of cDNA libraries. However, low abundance of the starting material and many cycles of PCR amplification often incorporate errors into the nucleotide sequence (Keohavong et al., "Fidelity of DNA Polymerases in DNA Amplification," Proc. Natl. Acad. Sci. U.S.A., 86:9253-9257 (1989) and Saiki et al., "Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," Science, 239:487-491 (1988), which are hereby incorporated by reference). In the present study, only UNI-AMP™ PCR amplified cDNA was used as a probe for screening. The cDNA library was generated by direct cloning of the cDNA that was synthesized by AMV reverse transcriptase. Therefore, the cDNA cloned inserts are believed to more accurately reflect the actual sequence of the dsRNA and the genomic RNA of GLRaV-3.

A total of 15,227 nucleotides or about 80% of the estimated 16 kb GLRaV-3 dsRNA was cloned and sequenced. Identification of this sequence fragment as the GLRaV-3 genome was based on its sequence alignment with the coat protein gene of GLRaV-3. This is the first direct evidence showing that high molecular weight dsRNA (~16 kb) isolated from GLRaV-3 infected vines is derived from GLRaV-3 genomic RNA. Based upon the nine ORFs identified, the genome organization of GLRaV-3

bears significant similarity to the other closteroviruses sequenced (BYV, CTV, and LIYV) (Figure 19).

Dolja (1994), which is hereby incorporated by reference, divided the closterovirus genome into four modules. For GLRaV-3, the 5′ accessory module including protease and vector transmission factor is yet to be identified. The core module, including key domains in RNA replication machinery (MET-HEL-RdRp) that is conserved throughout the alphavirus supergroup, has been revealed in parts of the HEL and RdRp domains. The MET domain has not yet been identified for GLRaV-3. The chaperon module, including three ORFs coding for the small transmembrane protein, the HSP70 homologue, and the distantly related HSP90 homologue, has been fully sequenced. The last module includes coat protein and its possible diverged copy and is also preserved in GLRaV-3. Overall similarity of the genome organization of GLRaV-3 with other closteroviruses further support the inclusion of GLRaV-3 as a member of closteroviruses (Hu (1990) and Martelli (1991), which are hereby incorporated by reference). However, observation of a ambisense gene on its 3′ terminal region may separate GLRaV-3 from other closteroviruses. Further comparative sequence analysis (Table 3) as well as phylogenetic observation of GLRaV-3 with respect to other closteroviruses over the entire genome sequence region suggested that GLRaV-3 is most closely related to BYV, followed by CTV, and LIYV.

As suggested by others (Agranovsky (1994), Dolja (1994), Karasev (1995), and Klaassen (1995), which are hereby incorporated by reference), expression of ORF 1b in closteroviruses may be via a +1 ribosomal frameshift mechanism. In GLRaV-3, a potential translation frameshift of ORF 1b could make a fusion

HEL-RdRp protein of over 1,926 amino acid residues with a capacity to encode a protein of more than 210K Comparative study of GLRaV-3 with respect to other closteroviruses over the ORF 1a/1b overlap revealed a significant sequence similarity to LIYV, but not to BYV or to CTV. The so-called slippery sequence (GGGUUU) and stem-loop and pseudoknot structures identified in BYV (Agranovsky (1994), which is hereby incorporated by reference) is not present in GLRaV-3. Thus, a frameshift mechanism that is similar to LIYV may be employed for GLRaV-3. However, protein analysis is necessary in order to determine the protein encoding capacities of these ORFs.

Differing from BYV, both CTV and LIYV have an extra ORF (ORF 2) in between RdRp (ORF 1b) and the small membrane protein (ORF 3) and potentially encoding a protein of 33K or 32K, respectively. However, in GLRaV-3, there is a much smaller ORF 2 (7K) followed by a long intergenic region of 1,065 bp. Thus, nucleotide sequencing of additional clones around this region may be necessary to resolve this discrepancy.

So far, among all plant viruses described, the HSP70 related gene is present only in the closteroviruses (Dolja (1994), which is hereby incorporated by reference). Identification of the GLRaV-3 HSP70 gene was based on an assumption that this gene should also be present in the closterovirus associated with grapevine leafroll disease, specifically GLRaV-3. Thus, cDNA clones that reacted with HSP70-degenerated primers were identified for sequence analysis. The identification of subsequent clones for sequencing was based on the gene-walking methodology. However, identification of immunopositive clones enabled identification of the coat protein gene of GLRaV-3 and

proved that the HSP70-containing sequence fragment is present in the GLRaV-3 RNA genome.

The 16 kb dsRNA used for cDNA synthesis was assumed to be a virus replicative form (Hu (1990), which is hereby incorporated by reference). Identification of the virus coat protein from this study further supports this assumption. Several lines of evidence show that the partial genome of GLRaV-3 has been cloned and sequenced. First, selected clones have been shown by Northern hybridization to hybridize to the 16 kb dsRNA and several smaller RNAs (presumably subgenomic RNAs) (Figure 4). Second, three GLRaV-3 antibody-reacting clones were identified after immuno-screening of the protein expressive library with both GLRaV-3 polyclonal (Zee (1987), which is hereby incorporated by reference) and monoclonal (Hu (1990), which is hereby incorporated by reference) antibodies. After nucleotide sequencing, these three antibody-reacting clones were shown to overlap one another and contain a common ORF which potentially encodes a protein with calculated *Mr* of 35K. This is in general agreement with the *Mr* estimated on SDS-PAGE (41K). Third, analysis of the partial genome sequence of GLRaV-3 suggested a close similarity in genome organization and gene sequences to the other closteroviruses (Dolja (1994), which is hereby incorporated by reference).

Information regarding the genome of GLRaV-3 provides a better understanding of this and related viruses and adds to the fundamental knowledge of closteroviruses. Present work on the nucleotide sequence and genome organization (about 80% of the estimated genome sequence) has provided direct evidence of a close relationship between GLRaV-3 and other closteroviruses. It has also made it possible, for the first time, to thoroughly evaluate a phylogenetic

relationship of GLRaV-3 based on a wide range of genes and gene products (helicase, polymerase, HSP70 homologue, HSP90 homologue, and coat protein). Based upon major differences in genome format and organization between BYV, CTV, and LIYV, along with phylogenetic analysis, Dolja (1994), which is hereby incorporated by reference, proposed the establishment of the new family *Closteroviridae* with three new genera of *Closterovirus* (BYV), *Citrivirus* (CTV), and *Biclovirus* (LIYV). This work on genome organization and phylogenetic analysis, along with evidence that this virus is transmitted by mealybugs (Engelbrecht et al., "Association of a Closterovirus with Grapevines Indexing Positive for Grapevine Leafroll Disease and Evidence for its Natural Spread in Grapevines," Phytopathol. Mediter., 24:101-105 (1990), Engelbrecht et al., "Field Spread of Corky Bark Fleck Leafroll and Shiraz Decline Diseases and Associated Viruses in South African Grapevines," Phytophylactica, 22:347-354 (1990), Engelbrecht et al., "Transmission of Grapevine Leafroll Disease and Associated Closteroviruses by the Vine Mealybug Planococcus-Ficus," Phytophylactica, 22:341-346 (1990), Rosciglione et al., "Transmission of Grapevine Leafroll Disease and an Associated Closterovirus to Healthy Grapevine by the Mealybug Planococcus Ficus (Abstract)," Phytoparasitica, 17:63-63 (1989), and Tanne et al., "Transmission of Closterolike Particles Associated with Grapevine Leafroll by Mealybugs (Abstract)," Phytoparasitica, 17:55 (1989), which are hereby incorporated by reference), suggest that a new genus under *Closteroviridae* family should be established. Thus, GLRaV-3 (the NY1 isolate) is proposed to be the type representative of the new genus, *Graclovirus* (*gra*-pevine *clo*-sterovirus). Further sequencing of other

grapevine leafroll associated closteroviruses may add more members to this genus.

Another cDNA library of GLRaV-3 has been established recently from dsRNA of an Italian isolate of GLRaV-3 (Saldarelli et al., "Detection of Grapevine Leafroll-Associated Closterovirus III by Molecular Hybridization," Plant Pathology (Oxford), 43:91-96 (1994), which is hereby incorporated by reference). Selected clones react specifically to GLRaV-3 dsRNA on a Northern blot; however, no direct evidence was provided to suggest that those clones were indeed from GLRaV-3 genomic RNA. Meanwhile, a small piece of sequence information from one of those cDNA clones was used to synthesize primers for the development of a PCR detection method (Minafra (1994), which is hereby incorporated by reference). Direct sequence comparison of these primer sequences to GLRaV-3 genome sequence obtained in the present study, showed that one of the primers (H229, 5'A-T-A-A-G-C-A-T-T-C-g-G-G-A-T-G-G-A-C-C (SEQ. ID. No. 27)) is located at nucleotides 5562-5581 and the other (C547, 5'A-T-T-A-A-C-t-T-g-A-C-G-G-A-T-G-G-C-A-C-G-C (SEQ. ID. No. 28)) is in reverse direction and is the complement of nucleotides 5880-5901. Mismatching nucleotides between the primers and GLRaV-3 sequence are shown in lowercase letters. Sequence comparison over these short primer regions to GLRaV-3 (isolate NY1) genome sequence showed a 90-95% identity, which suggested that these two isolates belong to the same virus (GLRaV-3). Moreover, the primers prepared by Minafra (1994), which is hereby incorporated by reference, from the Italian isolate of GLRaV-3 produced an expected size of PCR product with templates prepared from the NY1 isolate of GLRaV-3.

The reminder of the GLRaV-3 genome can be sequenced using the methods described herein.

Example 14 - Identification and Characterization of the 43 K ORF

The complete nucleotide sequence of the GLRaV-3 HSP90 gene is given in Figure 18. Initial sequencing work indicated that a open reading frame ("ORF") potentially encoding for a protein with a calculated Mr of 43K (Figure 29) was downstream of the HSP70-related gene. This gene was selected for engineering because the size of its encoded product is similar to the GLRaV-3 coat protein gene. However, after sequence editing, this incomplete ORF was proven to be located in the 3' terminal region of the HSP90-related gene. It is referred to herein as the incomplete GLRaV-3 HSP90 gene or as the 43K ORF.

Example 15 - Custom-PCR Engineering the Incomplete GLRaV-3 HSP90 Gene for Expression in Plant Tissues

Two custom synthesized oligonucleotide primers, 5' primer (93-224, t-a-c-t-t-a-t-c-t-a-g-a-a-c-c-A-T-G-G-A-A-G-C-G-A-G-T-C-G-A-C-G-A-C-T-A (SEQ. ID. No. 29)) and 3' complimentary primer (93-225, t-c-t-t-g-a-g-g-a-t-c-c-a-t-g-g-A-G-A-A-A-C-A-T-C-G-T-C-G-C-A-T-A-C-T-A (SEQ. ID. No. 30)) that flank the 43K ORF were designed to amplify the incomplete HSP90 gene fragment by polymerase chain reaction ("PCR"). Addition of a restriction enzyme *Nco* I site in the primer is for the convenience of cloning and for protein expression (Figure 29) (Slightom, "Custom Polymerase-Chain-Reaction Engineering of a Plant Expression Vector," Gene, 100:251-255 (1991), which is hereby incorporated by reference). Using these primers, a product of the proper size (1.2 kb) was amplified by reverse transcription PCR ("RT-PCR") using GLRaV-3 double-stranded RNA ("dsRNA") as template. The PCR amplified product was treated with *Nco* I, isolated from a low-

melting temperature agarose gel, and cloned into the same restriction enzyme treated binary vector pBI525 (obtained from William Crosby, Plant Biotechnology Institute, Saskatoon, Sask., Canada), resulting in a clone pBI525GLRaV-3hsp90 (Figure 30). A plant expression cassette, the *Eco*R I and *Hind* III fragment of clone pBI525GLRaV-3hsp90, which contains proper engineered CaMV 35S promoters and a Nos 3' untranslated region, was excised and cloned into a similar restriction enzyme digested plant transformation vector, pBin19 (Figure 30) (Clontech Laboratories, Inc.). Two clones, pBin19GLRaV-3hsp90-12-3 and pBin19GLRaV-3hsp90-12-4 that were shown by PCR to contain the proper size of the incomplete HSP90 gene were used to transform the avirulent *Agrobacterium tumefaciens*, strain LBA4404 via electroporation (Bio-Rad). The potentially transformed *Agrobacterium* was plated on selective media with 75 μg/ml of kanamycin. *Agrobacterium* lines which contain the HSP90 gene sequence were used to transform tobacco (*Nicotiana tobaccum* cv.Havana 423) using standard procedures (Horsch et al., "A Simple and General Method for Transferring Genes into Plants," Science, 227:1229-1231 (1985) ("Horsch (1985)"), which is hereby incorporated by reference). Kanamycin resistant tobacco plants were analyzed by PCR for the presence of the transgene. Transgenic tobacco plants with the transgene were self pollinated and seed was harvested.

Example 16 - Custom-PCR Engineering of the 43K ORF

The complete sequence of the GLRaV-3 hsp90 gene was reported in Figure 18. However, in the present study, using two custom synthesized oligo primers (93-224, tacttatctagaaccATGGAAGCGAGTCGACGACTA (SEQ. ID. No. 29) and 93-225, tcttgaggatccatggAGAAACATCGTCGCATACTA (SEQ. ID. No. 30)) and GLRaV-3 dsRNA as template, the

incomplete HSP90 related gene sequence was amplified by RT-PCR which added an *Nco* I restriction enzyme recognition sequence (CCATGG) around the potential translation initiation codon (ATG) and another *Nco* I site, 29 nt downstream from the translation termination codon (TAA) (Figure 29). The PCR amplified fragment was digested with *Nco* I, and cloned into the same restriction enzyme treated plant expression vector, pBI525. Under ampicillin selective conditions, hundreds of antibiotic resistant, transformants of *E. coli* strain DH5a were generated. Clones derived from five colonies were selected for further analysis. Restriction enzyme mapping (*Nco* I or *Bam*H I and *Eco*R V) showed that three out of five clones contained the proper size of the incomplete GLRaV-3 HSP90 sequence. Among them, two clones were engineered in the correct 5'-3' orientation with respect to the CaMV-AMV gene regulatory elements in the plant expression vector, pBI525. A graphical structure in the region of the plant expression cassette of clone pBI525GLRaV-3hsp90-12 is presented in Figure 30.

The GLRaV-3 HSP90 expression cassette was removed from clone pBI525GLRaV-3hsp90-12 by a complete digestion with *Hind* III and *Eco*R I and cloned into the similar restriction enzyme treated plant transformation vector pBin19. A clone designated as pBin19GLRaV-3hsp90-12 was then obtained (Figure 30) and was subsequently mobilized into the avirulent *Agrobacterium* strain LBA4404 using a standard electroporation protocol (Bio-Rad). Potentially transformed Agrobacteria were then plated on a selective medium (75 μg/ml kanamycin), and antibiotic resistant colonies were analyzed further by PCR with specific synthesized primers (93-224 and 93-225) to see whether or not the incomplete HSP90 gene was still present. After

analysis, clone LBA4404/pBin19GLRaV-3hsp90-12 was selected and used to transform tobacco tissues.

Example 17 - Transformation and Characterization of Transgenic Plants

The genetically engineered *Agrobacterium tumefaciens* strain, LBA4404/pBin19GLRaV-3hsp90-12, was co-cultivated with tobacco leaf discs as described (Horsch (1985), which is hereby incorporated by reference). Potentially transformed tobacco tissues were selected on MS regeneration medium (Murashige et al., "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures," Physiologia Plantarum, 15:473-497 (1962), which is hereby incorporated by reference) containing 300 μg/ml of kanamycin. Numerous shoots developed from kanamycin resistant calli in about 6 weeks. Rooted tobacco plants were obtained following growth of developed shoots on a rooting medium (MS without hormones) containing 300 μg/ml of kanamycin. Eighteen independent, regenerated kanamycin resistant plants were transplanted in a greenhouse and tested for the presence of the HSP90-related gene by PCR. Fourteen out of eighteen selected kanamycin resistant transgenic lines were shown to contain a PCR product with the expected size (Figure 31).

The genetically engineered *Agrobacterium tumefaciens* strain LBA4404/pBin19GLRaV-3hsp90-12 was also used to transform the grapevines rootstock Couderc 3309 (*Vitis riparia* x *Vitis rupestris*). Embryogenic calli of Couderc 3309 were obtained by culturing anthers on MSE media. (MSE media contained Murashige and Skoog salts plus 0.2% sucrose, 1.1 mg/L 2,-4-D, and 0.2 mg/L BA. The media were adjusted to pH 6.5, and 0.8% Noble agar was added. After autoclaving, 100 ml M-0654, 100 ml M-0529, and 1 ml vitamin M-3900 were added to the

media). After 60 days, primary calli were induced and transferred to hormone-free HMG medium (1/2 Murashige salts with 10 g/L sucrose, 4.6 g/L glycerol and 0.8% Noble agar) for embryogenesis. Calli with globular or heart-shaped embryos were immersed for 15 minutes in *Agrobacterium tumefaciens* LBA4404/pBin19GLRaV-3hsp90-12 that was suspended in MS liquid medium. The embryos were blotted on filter paper to remove excess liquid and transferred to HMG medium with acetosyringone (100 μM) and kept for 48 hours in the dark at 28°C. The calli were then washed 2-3 times in MS liquid medium plus cefotaxime (300 μg/ml) and carbenicillin (200 μg/ml) and transferred to HMG medium with the same antibiotics for 1-2 weeks. Subsequently, the embryogenic calli were transferred to HMG medium containing 20 or 40 mg/L kanamycin and 300 mg/L cefotaxime plus 200 mg/L carbenicillin to select transgenic embryos. After being on selection medium for 3-4 months, growing embryos were transferred to HMG, MGC (full-strength MS salts amended with 20 g/L sucrose, 4.6 g/L glycerol, 1 g/L casein hydrolysate, and 0.8% Noble agar), or MSE medium with kanamycin. After 4 months, germinated embryos were transferred to baby food jars containing rooting medium, such as a woody plant medium described, for example, in Lloyd et al., "Commercially Feasible Micropropogation of Mountain Laurel. *Kalmia latifolia*, By Use of Shoot Tip Culture," Proc. Intl. Plant Prop. Soc., 30:421-427 (1981), which is hereby incorporated by reference, that was supplemented with 0.1 mg/L BA, 3 g/L activated charcoal and 1.5% sucrose. The pH was adjusted to 5.8 and Noble agar was added to 0.7%]. Plantlets with roots were transplanted to pots with artificial soil mix and grown in greenhouses. In this manner, 88 grapevine plants were transferred to the greenhouse. The 43K

protein gene has been detected by PCR in a number of them.

Using the methods described above, engineering of the incomplete HSP90 gene of GLRaV-3 into plant expression and transformation vectors has been effected. The targeted gene sequence was shown to be integrated into the plant genome by PCR analysis of the transgenic tobacco plants. The engineered *Agrobacterium tumefaciens* strain LBA4404/pBin19GLRaV-3hsp90-12 has been used to transform grapes and tobacco. Furthermore, success in the genetic engineering of a plant transformation vector may serve as a model for further construction of other GLRaV-3 genes, such as coat protein, RdRp, and HSP70 that are now available.

Since the first demonstration of transgenic tobacco plants expressing the coat protein gene of TMV resulted in resistance against TMV infection (Powell-Abel et al., "Delay of Disease Development in Transgenic Plants that Express the Tobacco Mosaic Virus Coat Protein Gene," Science, 232:738-743 (1986), which is hereby incorporated by reference), the phenomenon of the coat protein-mediated protection has been observed for over 20 viruses in at least 10 different taxonomic groups in a wide variety of dicotyledonous plant species (Beachy et al., "Coat Protein-Mediated Resistance Against Virus Infection," Annu. Rev. Phytopathol., 28:451-74 (1990) ("Beachy (1990)") and Wilson, "Strategies to Protect Crop Plants Against Viruses: Pathogen-Derived Resistance Blossoms," Proc. Natl. Acad. Sci., U.S.A., 90:3134-3141 (1993) ("Wilson (1993)", which are hereby incorporated by reference). If gene silencing (or co-suppression) (Finnegan et al., "Transgene Inactivation: Plants Fight Back!" Bio/Technology, 12:883-888 (1994) and Flavell, "Inactivation of Gene Expression in Plants as a

Consequence of Specific Sequence Duplication," Proc. Natl. Acad. Sci. U.S.A., 91:3490-3496 (1994), which are hereby incorporated by reference) is one of the resistance mechanisms (Lindbo et al., "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance," The Plant Cell, 5:1749-1759 (1993), Pang et al., "Different Mechanisms Protect Transgenic Tobacco Against Tomato Spotted Wilt and Impatiens Necrotic Spot Tospoviruses," Bio/Technology, 11:819-824 (1993) ("Pang (1993)"), and Smith et al., "Transgenic Plant Virus Resistance Mediated by Untranslatable Sense RNAs: Expression, Regulation, and Fate of Nonessential RNAs," The Plant Cell, 6:1441-1453 (1994), which are hereby incorporated by reference), then one would expect to generate transgenic plants expressing any part of a viral genome sequence to protect plants from that virus infection. Thus, in the present study, trangenic plants expressing the 43K ORF (or the incomplete hsp90 gene) may be protected from GLRaV-3 infection.

Since tobacco (*Nicotiana tobaccum* cv. Havana 423) is not the host of GLRaV-3, direct evaluation of the virus resistance was not possible. However, recently, after a mechanical inoculation of *N. benthamiana* with grapevine leafroll infected tissue, Boscia (1995), which is hereby incorporated by reference, have recovered a long closterovirus from *N. benthamiana* which is probably GLRaV-2. Thus, it is believed that other types of grapevine leafroll associated closteroviruses can also be mechanically transmitted to *N. benthamiana*. If the 43K ORF from GLRaV-3 can also be transferred to *N. benthamiana*, it might be possible to evaluate the resistance of those plants against GLRaV-2 infection. However, the resistance of the transgenic grape rootstock Couderc

3309 against leafroll infection can be presently evaluated.

Example 18 - Coat Protein-mediated Protection and Other Forms of Pathogen-derived Resistance

The successful engineering technique used in the above work could be utilized to engineer other gene sequences of GLRaV-3 which have since been identified. Among these, the coat protein gene of GLRaV-3 is the primary candidate since coat protein-mediated protection (Beachy (1990), Hull et al., "Approaches to Nonconventional Control of Plant Virus Diseases," Crit. Rev. Plant Sci., 11:17-33 (1992), and Wilson (1993), which are hereby incorporated by reference) has been the most successful example in the application of the concept of pathogen-derived resistance (Sanford et al., "The Concept of Parasite-Derived Resistance - Deriving Resistance Genes from the Parasite's Own Genome," J. Theor. Biol., 113:395-405 (1985), which is hereby incorporated by reference). Construction of plant expression vector (pEPT8/cpGLRaV-3) and *Agrobacterium* binary vector (pGA482pEPT8/cpGLRaV-3) was done following a strategy similar to the above. The GLRaV-3 coat protein gene was PCR amplified with primers (KSL95-5, a-c-t-a-t-t-t-c-t-a-g-a-a-c-c-A-T-G-G-C-A-T-T-T-G-A-A-C-T-G-A-A-A-T-T (SEQ. ID. No. 31), and KSL95-6, t-t-c-t-g-a-g-g-a-t-c-c-a-t-g-g-T-A-T-A-A-G-C-T-C-C-C-A-T-G-A-A-T-T-A-T (SEQ. ID. No. 32)) and cloned into pEPT8 after *Nco*I treatment. The expression cassette from pEPT8/cpGLRaV-3 (including double CaMV 35S enhancers, 35S promotor, alfalfa mosaic virus leader sequence, GLRaV-3 coat protein gene, and 35S terminator) was digested with *Hin*dIII and cloned into pGA482G (Figure 32). Resulting *Agrobacterium* binary vector (pGA482GpEPT8/cpGLRaV-3) was mobilized into

Agrobacterium tumerfaciens strain C58Z707 and used for transformation of grapevines.

Other gene sequence (ORF 1b, the RNA dependent RNA polymerase) may also be used, as replicase-mediated protection has been effectively used to protect plants from virus infection (Carr et al., "Replicase-Mediated Resistance," Seminars in Virology, 4:339-347 (1993) and Golemboski et al., "Plants Transformed with a Tobacco Mosaic Virus Nonstructural Gene Sequence are Resistant to the Virus," Proc. Natl. Acad. Sci. U.S.A., 87:6311-6315 (1990), which are hereby incorporated by reference). The HSP70 homologue may also be used to generate transgenic plants that are resistant against all types of grapevine leafroll associated closteroviruses since significant consensus sequences are observed over HSP70 conserved domains. Moreover, the phenomenon of RNA-mediated protection has also been observed (de Haan et al., "Characterization of RNA-Mediated Resistance to Tomato Spotted Wilt Virus in Transgenic Tobacco Plants," Bio/Technology, 10:1133-1137 (1992), Farinelli et al., "Coat Protein Gene-Mediated Resistance to Potato Virus Y in Tobacco Examination of the Resistance Mechanisms is the Transgenic Coat Protein Required for Protection?" Mol. Plant Microbe Interact., 6:284-292 (1993) ("Farinelli (1993)"), Kawchuk et al., "Sense and Antisense RNA-Mediated Resistance to Potato Leafroll Virus in Russet Burbank Potato Plants," Mol. Plant Microbe Interact, 4:247-253 (1991) ("Kawchuk (1991)"), Lindbo et al., "Untranslatable Transcripts of the Tobacco Etch Virus Coat Protein Gene Sequence Can Interfere with Tobacco Etch Virus Replication in Transgenic Plants and Protoplasts," Virology, 189:725-733 (1992), Lindbo et al., "Pathogen-Derived Resistance to a Potyvirus Immune and Resistant Phenotypes in Transgenic Tobacco Expressing Altered Forms of a

Potyvirus Coat Protein Nucleotide Sequence," Mol. Plant Microbe Interact, 5:144-153 (1992), Lindbo et al., "Pathogen Derived Resistance to Potyviruses: Working, But Why?" Seminars in Virology, 4:369-379 (1993), Pang (1993), and Van Der Wilk et al., "Expression of the Potato Leafroll Luteovirus Coat Protein Gene in Transgenic Potato Plants Inhibits Viral Infection," Plant Mol. Biol., 17:431-440 (1991), which are hereby incorporated by reference). Thus, untranslatable transcript versions of the above mentioned GLRaV-3 genes might also produce leafroll resistant transgenic plants.

Another form of pathogen-derived resistance that has also been shown to be effective in control of plant viral disease is through the use of antisense RNA. Transgenic tobacco plants expressing the antisense sequence of the coat protein gene of cucumber mosaic virus ("CMV") showed a delay in symptom expression by CMV infection (Cuozzo et al., "Viral Protection in Transgenic Tobacco Plants Expressing the Cucumber Mosaic Virus Coat Protein or its Antisense RNA," Bio/Technology, 6:549-554 (1988), which is hereby incorporated by reference). Transgenic plants expressing either potato virus X ("PVX") coat protein or its antisense transcript were protected from infection by PVX. However, plants expressing antisense RNA were protected only at low inoculum concentration. The extent of this protection mediated by antisense transcript is usually lower than transgenic plants expressing the coat protein (Hemenway et al., "Analysis of the Mechanism of Protection in Transgenic Plants Expressing the Potato Virus X Coat Protein or its Antisense RNA," Embo. (Eur. Mol. Biol. Organ.) J., 7:1273-1280 (1988), which is hereby incorporated by reference). This type of resistance has also been observed in bean yellow mosaic virus (Hammond et al.,

"Expression of Coat Protein and Antisense RNA of Bean Yellow Mosaic Virus in Transgenic Nicotiana-Benthamiana," Phytopathology, 81:1174 (1991), which is hereby incorporated by reference, tobacco etch virus (Lindbo et al., "Untranslatable Transcripts of the Tobacco Etch Virus Coat Protein Gene Sequence Can Interfere with Tobacco Etch Virus Replication in Transgenic Plants and Protoplasts," Virology, 189:725-733 (1992), which is hereby incorporated by reference), potato virus Y (Farinelli (1993), which is hereby incorporated by reference), and zucchini yellow mosaic virus (Fang et al., "Genetic Engineering of Potyvirus Resistance Using Constructs Derived from the Zucchini Yellow Mosaic Virus Coat Protein Gene," Mol. Plant Microbe Interact., 6:358-367 (1993), which is hereby incorporated by reference). However, high level of resistance mediated by antisense sequence was observed to be similar to potato plants (Russet Burbank) expressing potato leafroll virus coat protein (Kawchuk (1991), which is hereby incorporated by reference). Besides using antisense transcript of the virus coat protein gene, other virus genome sequences have also been demonstrated to be effective. These included the 51-nucleotide sequences near the 5' end of TMV RNA (Nelson et al., "Tobacco Mosaic Virus Infection of Transgenic Nicotiana-Tabacum Plants is Inhibited by Antisense Constructs Directed at the 5' Region of Viral RNA," Gene (Abst), 127:227-232 (1993), which is hereby incorporated by reference) and noncoding region of turnip yellow mosaic virus genome (Zaccomer et al., "Transgenic Plants that Express Genes Including the 3' Untranslated Region of the Turnip Yellow Mosaic Virus (TYMV) Genome are Partially Protected Against TYMV Infection," Gene, 87-94 (1993), which is hereby incorporated by reference).

GLRaV-3 has been shown to be transmitted by mealybugs and in some cases it has been shown to spread rapidly in vineyards (Engelbrecht et al., "Field Spread of Corky Bark Fleck Leafroll and Shiraz Decline Diseases and Associated Viruses in South African Grapevines," Phytophylactica, 22:347-354 (1990), Engelbrecht et al., "Transmission of Grapevine Leafroll Disease and Associated Closteroviruses by the Vine Mealybug Planococcus-Ficus," Phytophylactica, 22:341-346 (1990), and Jordan et al., "Spread of Grapevine Leafroll and its Associated Virus in New Zealand Vineyards," 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, pp. 113-114 (1993), which are hereby incorporated by reference). This disease may become more of a problem if mealybugs become difficult to control due to the lack of insecticides. In this scenario, the development of leafroll resistant grapevines becomes very attractive. Although grapevine is a natural host of *Agrobacterium* (*A. vitis* is the causal agent of the grapevine crown gall disease), transformation of grapevine has proven to be difficult (Baribault et al., "Transgenic Grapevines: Regeneration of Shoots Expressing β-glucuronidase," J. Exp. Bot., 41:1045-1049 (1990), Baribault et al., "Genetic Transformation of Grapevine Cells," Plant Cell Reports, 8:137-140 (1989), Colby et al., "Cellular Differences in *Agrobacterium* Susceptibility and Regenerative Capacity Restrict the Development of Transgenic Grapevines," J. Am. Soc. Hort. Sci., 116:356-361 (1991), Guellec et al., "Agrobacterium-Rhizogenes Mediated Transformation of Grapevine Vitis- Vinifera 1, Agrobacterium-Rhizogenes Mediated Transformation of Grapevine Vitis- Vinifera 1," Plant Cell Tissue Organ Cult., 20:211-216 (1990), Hebert et al., "Optimization of Biolistic Transformation of Embryogenic Grape Cell Suspensions," Plant Cell Reports,

12:585-589 (1993), Le Gall et al., "Agrobacterium-Mediated Genetic Transformation of Grapevine Somatic Embryos and Regeneration of Transgenic Plants Expressing the Coat Protein of Grapevine Chrome Mosaic Nepovirus (GCMV)," Plant Science, 102:161-170 (1994), Martinelli et al., "Genetic Transformation and Regeneration of Transgenic Plants in Grapevine (Vitis Rupestris S.)," Theoretical and Applied Genetics, 88:621-628 (1994), and Mullins et al., "*Agrobacterium*-Mediated Genetic Transformation of Grapevines: Transgenic Plants of *Vitis rupestris* Scheele and Buds of *Vitis vinifera* L.," Bio/Technology, 8:1041-1045 (1990), which are hereby incorporated by reference). Recently, an efficient regeneration system using proliferative somatic embryogenesis and subsequent plant development has been developed from zygotic embryos of stenospermic seedless grapes (Mozsar, J. et al., "A Rapid Method for Somatic Embryogenesis and Plant Regeneration from Cultured Anthers of Vitis Riparia," Vitis, 33:245-246 (1994), and Emerschad (1995), which are hereby incorporated by reference). Using this regeneration system, Scorza et al., "Transformation of Grape (Vitis vinifera L.) Zygotic-Derived Somatic Embryos and Regeneration of Transgenic Plants," Plant Cell Reports, 14:589-592 (1995) ("Scorza (1995)"), which is hereby incorporated by reference, succeeded in obtaining transgenic grapevines through zygotic-derived somatic embryos after particle-wounding/*A. tumefaciens* treatment. Using a Biolistic device, tiny embryos were shot with gold particles (1.0 μm in diameter). The wounded embryos were then co-cultivated with *A. tumefaciens* containing engineered plasmids carrying the selection marker of kanamycin resistance and β-glucuronidase ("GUS") genes. Selection of transgenic grapevines was carried out with 20 μg/ml kanamycin in the initial stage and then 40

μg/ml for later proliferation. Small rooted seedlings were obtained from embryogenic culture within 5 months of bombardment/*A. tumefaciens* (Scorza (1995), which is hereby incorporated by reference). Transgenic grapevines were analyzed by PCR and Southern hybridization, and shown to carry the transgenes. The above-mentioned grapevine transformation approach has been carried out in the current investigation to generate transgenic grapevines expressing GLRaV-3 genes. Evaluation of any potential leafroll resistance on transgenic grapevines may be carried out by insect vectors or grafting.

Example 19 - Production of Antibodies Recognizing GLRaV3

The clone pCP10-1 which was shown to contain the major portion of the coat protein gene of GLRaV3 (Figure 9) was used to express the coat protein and the β-galactosidase fusion protein. About 500 ml of LB medium containing 50 μg/ml of ampicilian was inoculated with a pCP10-1 single colony and incubated with rigorous shaking for overnight until log-phase growth. Expression of the fusion protein was further induced by the addition of 1 mM IPTG. Bacteria were harvested by centrifugation at 5,000 rpm for 10 min. The bacterial cell wall was broken by sonication. After low speed centrifugation to get rid of cell debris, the fusion protein was precipitated by the addition of saturated ammonium sulfate, then resuspended in PBS buffer and electrophoresced in a SDS-polyacrylamide gel ("SDS-PAGE"). The fusion protein band was excised after soaking the SDS-PAGE gel in 0.25M KCl to locate the protein band. The protein was eluted with buffer (0.05M Tris-HCl, pH7.9, 0.1% SDS, 0.1 mM EDT and 0.15M NaCl) and precipitated by trichoroacetic acid to a final concentration of 20%.

An antiserum was prepared by immunization of a rabbit with 0.5-1 mg of the purified protein emulsified with Freund's completed adjuvant followed by two more weekly injections of 0.5-1 mg protein emulsified with Freund's incomplete adjuvant. After the last injection, antisera were collected from blood taken from the rabbit every week for a period of 4 months.

On Western blot analysis, the antibody gave a specific reaction to the 41K protein from GLRaV3 infected tissue as well as to the fusion protein itself (50K) and generated a pattern similar to the pattern seen in Figure 8. This antibody was also successfully used as a coating antibody and as an antibody-conjugate in enzyme linked immunosorbent assay ("ELISA").

The above method of producing antibody to GLRaV3 can also be applied to other gene sequences of the present invention. The method affords a large amount of highly purified protein from *E. coli* from which antibodies can be readily obtained. It is particularly useful in the common case where it is rather difficult to obtain sufficient amount of purified virus from GLRaV3 infected grapevine tissues.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.